The Molecular Basis of Biocide Resistance and Susceptibility in Bacteria

Thesis presented for the Degree of Philosophiae Doctor

By

Saber Yezli, BSc (Hons) Genetics

In Candidature for the Degree of Philosophiae Doctor

Cardiff School of Biosciences

Cardiff University

December 2007

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

- I wish to thank my supervisor Dr Eshwar Mahenthiralingam, Cardiff School of Biosciences, for his outstanding help, supervision, and expert guidance throughout this study. I would also like to acknowledge my other supervisor Prof Denver Russell, Welsh School of Pharmacy, Cardiff, who died in September 2004, for his invaluable contribution to this work.

- I wish to thank Advanced Sterilization Products, Johnson & Johnson for providing the funding for this study.

- I would like to thank my parents, my sisters Sabra and Baya and my brother Ali for their support and encouragement.

- I would like to thank Olga for her love, understanding and encouragement the entire duration of this study.

- Special thanks to my friends, colleagues and staff at Cardiff University School of Biosciences for their help, technical assistance and friendship.

CONFERENCES AND PRESENTATIONS

- **Yezli, S., A. D. Russell, and E. Mahenthiralingam.** 2006. **The Molecular Basis of Biocide Resistance and Susceptibility in *Serratia*.** Abstracts of the 106th Annual Meeting of the American Society for Microbiology (ASM): Abstract A-012.

- **The Molecular Basis of Biocide Resistance and Susceptibility in *Serratia*.** 4th All Wales Microbiology Meeting. March, 2006. Gregynog, Wales, UK.

The Molecular Basis of Biocide Resistance and Susceptibility in Bacteria

CONTENTS

CHAPTER III

Molecular basis of biocide resistance and susceptibility in *Serratia*

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABC	ATP binding cassette
AHL	*N*-acyl-homoserine lactone
ATCC	American type culture collection
AMK	Amikacin
AMP	Ampicillin
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AZM	Azithromycin
BAC	Benzalkonium chloride
Bcr	Bicyclomycin resistance protein
BLAST	Basic local alignment software tool
BSAC	British society for antimicrobial chemotherapy
bp	Base pair
Ca^{2+}	Calcium ion
cAMP	Adenosine 3′,5′-cyclic monophosphate
CAZ	Ceftazidime
CDNB	Chlorodinitrobenzene
CHL	Chloramphenicol
CFU	Colony forming unit
CH_2O	Formaldehyde
CHX	Chlorhexidine diacetate
CIP	Ciprofloxacin
CLR	Clarithromycin
CLSI	Clinical and laboratory standards institute
CPC	Cetylpyridinium chloride
CRP	cAMP receptor protein
CSF	Colony stimulated growth factor
CSPD	Disodium 3(4-methoxispiro[1,2,dioxetane-3,2-{5'Chloro}Tricyclo.3.1.1^{3,7}]decan)-4-yl phenyl phosphate
Da	Dalton
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DMT	Drug/metabolite transporter superfamily
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
E-test	Epsilometer test
EDTA	Ethylenediaminetetraacetic acid
EMB	Ethambutol
EMS	Ethylmethanesulfonate
Ept	Efficient plasmid transformation
ETH	Ethionamide
EUCAST	European committee on antimicrobial susceptibility testing
FAS	Fatti acid synthase
FDA	Food and drug administration
FOX	Cefoxitin
FQ	Fluoroquinolone

G+C content	Guanine + cytosine content
GDP	Guanosine 5′-diphosphate
GEN	Gentamicin
GLC	Gas-liquid chromatography
GlcN-Ins	1-Dmyo-inosityl-2amino-2-deoxy-α-D-glucopyranoside
GTA	Glutaraldehyde
H_2O	Water
H_2O_2	Hydrogen peroxide
H-NS	Histone-like nucleoid structuring protein
HPLC	High performance liquid chromatography
HSL	Homoserine lactone
HSP	Heat shock protein
IEF	Isoelectric focusing
IMPase	Inositol monophosphate phosphatase
IPM	Imipenem
INH	Isoniazid
IS	Insertion sequence
IWGMT	International working group on mycobacterial taxonomy
KAN	Kanamycin
KCl	Potassium chloride
kDa	Kilodaltons
KH_2PO_4	Potassium hydrogen phosphate
LAM	Lipoarabinomannan
LB	Luria-Bertani
mAG	Mycolyl-arabinogalactan
mAGP	Mycolyl-arabinogalactan-peptidoglycan complex
MAP	2-Methyl-3-amylpyrrole
MATE	Multidrug and toxic compound extrusion family
mBBr	Monobromobimane
MBC	Minimal bactericidal concentration
MBC	4-Methoxy-2,2'bipyrrole-5-carboxyaldehyde
MEM	Meropenem
MFS	Major facilitator superfamily
Mg^2	Magnesium ion
$MgCl_2$	Magnesium Chloride
$MgSO_4.7H_2O$	Magnesium sulphate
MIC	Minimal inhibitory concentration
Mn-SOD	Manganese superoxide dismutase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSH	Mycothiol
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NaCl	Sodium chloride
NCTC	National collection of type cultures
NCCLC	National committee for clinical laboratory standards
NTG	N-methyl-N'-nito-N-nitrosoguanidine
NTM	Nontuberculous mycobacteria
OADC	Oleic acid-albumin-dextrose-catalase
OD	Optical density
OFX	Ofloxacin

OHHL	*N*-(3-oxohexanoyl)-homoserine lactone
Omp	Outer membrane protein
OPA	*Ortho*-phthalaldehyde
ORF	Open reading frame
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PET	Putative efflux transporter
Pfam	Protein family
PHMB	Polyhexamethylene biguanide
PIP	Piperacillin
PIMs	Phosphatidylinositol mannosides
PMF	Proton motive force
PPM	Parts per million
PQS	2-heptyl-3-hydroxy-4-quinolone
PRA	PCR-restriction fragment length polymorphism analysis
PXB	Polymyxin B
PZA	Pyrazinamide
QACs	Quaternary ammonium compounds
QRDR	Quinolone resistance-determining region
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RGM	Rapidly growing mycobacteria
RIF	Rifampicin
RNA	Ribonucleic acid
RNase	Ribonuclease
RND	Resistance-nodulation-division family
rpm	Revolution per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SGM	Slow growing mycobacteria
SMR	Small multidrug resistance
SPX	Sparfloxacin
SSC	Sodium chloride-sodium citrate
STR	Streptomycin
SXT	Trimethoprim/sulfamethoxazole
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TIGR	The institute for genomic research
TNF	Tumour necrosis factor
TOB	Tobramycin
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UDP	Uridine 5′-diphosphate.
UV	Ultraviolet
VOC	Vicinal oxygen chelate

V/V	Volume/volume
W/V	Weight/volume
W/W	Weight/ weight
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
$ZnSO_4$	Zinc sulfate

SUMMARY

The molecular basis of biocide resistance and susceptibility in *Serratia* and mycobacteria was investigated using transposon mutagenesis approach. The killing and growth inhibitory effects of four biocides (triclosan, cetylpyridinium chloride, chlorhexidine diacetate and alkaline *ortho*-phthalaldehyde) on *Serratia marcescens* Db11, *Mycobacterium smegmatis* mc^2155, *M. chelonae* type strain NCTC 946, *M. abscessus* type strain ATCC 19977, and *Escherichia coli* NCTC 1048 were studied using minimal inhibitory concentration determination, biocide killing, and potassium leakage tests. Transposon mutagenesis using a *mariner* system did not produce any *M. smegmatis* mc^2155 mutants with altered biocide sensitivity. In contrast mutagenesis of *S. marcescens* Db11 using the mini-Tn*5*Km2 transposon system led to the isolation of 26 biocide mutants. Increased resistance, susceptibility and mixed biocide phenotypes were observed in the mutants. Alteration in antibiotic susceptibility was also noted. The locations of transposon insertion in all but two of the mutants were determined, and 14 putative genes coding for putative proteins with diverse functions were found to be disrupted. These functions included anabolism and catabolism, gene regulation, cell envelope biosynthesis, porin, energy production, and virulence. Two mutants, one deficient in the outer membrane protein A (OmpA), and another deficient in the nucleoid-associated protein (NdpA), were complemented. Complementation of the *ndpA* mutant which showed increased resistance to cetylpyridinium chloride and chlorhexidine diacetate, but was sensitive to triclosan, lead to restoration of the wild type phenotype. Complementation of the *ompA* mutant, which showed multiple sensitivity to chlorhexidine diacetate, triclosan, and *ortho*-phthalaldehyde however, did not restore the wild type phenotype. The cloned *ompA* gene was shown to be transcribed but not translated in the complemented mutant. In summary, the genetic basis for biocide resistance in *S. marcescens* Db11 is multi-factorial and encoded by several novel loci worthy of further study.

CHAPTER I

INTRODUCTION TO BIOCIDES

1.1 Overview

This chapter provides an introduction to biocides and will review the following:

- **Definition of the word "biocide"**
- **Mode of action of biocides**
- **Different classes of biocides**
- **Biocide resistance in bacteria**
- **Link between biocide and antibiotic resistance and development of cross-resistance**

1.2 Definition

The term "Biocide" is used to describe a chemical agent, usually broad-spectrum, that inactivates micro-organisms. Biocides include disinfectants, antiseptics and preservatives, but not antibiotics in spite the latter being biocides in the strictest sense (304). Antiseptics are used to destroy or inhibit the growth of micro-organisms in or on living tissue, whereas disinfectants are used on inanimate objects or surfaces. Preservatives are incorporated into pharmaceutical cosmetic and other products to prevent microbial contamination and multiplication (899). Biocides vary in the purpose of their use, some biocides have a mainly single usage such as glutaraldehyde which is used for disinfection, but others such as chlorhexidine and quaternary ammonium compounds (QACs) can be used as antiseptics, disinfectants, and preservatives. Moreover, biocides also differ in their antimicrobial activity, some are referred to as "static" able to inhibit growth, others are "cidal" which kill the target organism.

Biocides have been used for centuries mainly as preservative agents of drinking water, such as the use of copper and silver vessels, foodstuffs including slating and even in the art of mummification. Other agents with antimicrobial properties such as vinegar, wine, honey and mercuric chloride were used for wound dressing (646). The development of antiseptic surgery in the 19th century saw the introduction of disinfectant usage, with chemicals such as wood tar, copper sulphate, hydrogen peroxide and chlorine-releasing agents being used for infection control. Other agents such as QACs and chlorhexidine were introduced more recently (464). Nowadays there is a large number of chemicals used as biocides both as single agents and in complex formulations, and the scope of their use extends from hospitals and health care settings to industry to the home and domestic environment (464, 910).

Biocides fall into a number of families (described in section 1.4) that differ in their use and mode of action, in this study four key representatives of these families of biocides were investigated: *ortho*-phthalaldehyde (OPA), triclosan (TRI), cetylpyridinium chloride

(CPC), and chlorhexidine diacetate (CHX). The following will review the different types of biocide families and their use in both clinical and home environments. Description of their mode of action and development of resistance to them is also presented where literature reports are available.

1.3 Biocides mode of action

Biocides are classified on basis of their chemical structure, reactivity and mode of action. They vary greatly in their chemical structures and the precise mechanism(s) of their action often reflects this diversity. Moreover, there are many factors that influence biocide action and efficiency (915, 920), and these compounds are commonly used in complex formulations of active molecules, sometimes containing co-solvents, chelating agents, acidic or alkaline agents, or surface-active or anti-corrosive products. This makes their mechanism of action even more complex. Relatively few publications addressed the mechanism of inhibition and inactivation of Gram-positive non-sporulating bacteria, bacterial spores, and Gram-negative bacteria (456, 898). Even less is known about the mechanisms of fungal, protozoal and viral inactivation by biocides (647, 676, 1079).

Unlike most antibiotics, it is widely accepted that only few biocides exert their action upon one specific target within the microbial cell. Most agents have multiple target sites and the site of lethal action depends upon the concentration employed (920). Bacteriostatic effects are usually achieved by lower concentration of biocide, and might correspond to a reversible activity on the cytoplasmic membrane and/or effect on enzymatic activity. The bactericidal mechanism(s) of action of biocide is however less understood and a primary target site within the cell might be involved (646). In any case, for the biocide to be effective it has to reach and interact with its microbial target site(s). This interaction follows a similar sequence of events in which the biocide first binds to the surface of the cell. Subsequent changes in the outer cell layer may occur allowing the biocide to penetrate the cell wall and membrane reaching the cytoplasm, where it can interact with cellular proteins or nucleic acids. Alteration or damage to the bacterial structure at the outer layer, cytoplasmic membrane or within the cytoplasm at any stage

of the biocide-bacterial interaction may contribute to the bacteriostatic or bactericidal effect of the biocide (577, 646).

The overall mechanism of biocides action can be defined according to the bacterial structure or site against which it is most active. Three interaction sites have been described: (i) the outer cellular components or cell wall; (ii) the cytoplasmic membrane and its constituents and; (iii) the cytoplasm and its components. A biocide can act on one, two, or all three sites to achieve antimicrobial activity.

1.3.1 Action on the cell wall and outer cellular components

Several biocides are known to interact with outer cellular component of bacteria although cell viability might not be affected. The effect of this interaction may be changes in cell hydrophobicity and permeability. For instance, cationic agents such as chlorhexidine and QACs were shown to alter the hydrophobicity of Gram-negative bacteria (262, 263, 296) and damage the cell wall and outer membrane promoting their own uptake to reach their target(s) site at the cell cytoplasmic membrane or cytoplasm (343). Other biocides, such as glutaraldehyde, bind covalently to the cell wall components, including peptidoglycan. The effect of these cross linking agents such as aldehydes is not always apparent in altered cell appearance but the function of the cell wall is affected (358, 646). Some agents such as metal ion chelating compounds, although might not show strong bactericidal activity, they appear to act specifically against the bacterial outer membrane, and might enhance the activity of biocides if they are used in combination with these agents (27, 646, 916). Examples include EDTA, polycations, lactoferrin, transferrin and polyphosphates which were all shown to increase cell permeability of Gram-negative bacteria (269, 646, 1083, 1084).

1.3.2 Action on the cytoplasmic membrane and its components

Biocides which are active at the cytoplasmic membrane level are referenced to as "membrane active agents". The result of such cytoplasmic membrane disruption can be

seen in the leakage of the intracellular components. Potassium leakage is one of the first indicators of biocide-induced membrane damage and occurs usually rapidly following exposure to the biocide (578). Phenols, cresols and their chlorinated derivatives including chlorocresol and *para*-chloro-*meta* xylenol, have all been shown to induce leakage of intracellular materials from bacteria (460, 506, 559). Similarly, QACs and biguanides such as chlorhexidine are believed to combine with the membrane phospholipids causing disruption of the cytoplasmic membrane (1042). Other agents, such as organic acids and esters, may also induce leakage of intracellular components, although they also have other effects on the bacterial cell (646).

Biocides acting on the cytoplasmic membrane level may also inhibit the energy processes in the cell by disrupting the proton motive force (PMF). The latter is an expression of the energised state of the bacterial membrane, and is composed of an electrical potential and proton gradient that is maintained across the cytoplasmic membrane of the cell (577). The PMF is involved in active transport, oxidative phosphorylation and ATP synthesis in bacteria (461, 714, 715). It is generated by oxidation-reduction reactions occurring during electron transport. A number of biocides have effect on the PMF including some lipid-soluble phenols such as 2,4-dinitrophenol, and the protonophore, carbonyl cyanide-3-chlorophenylhydrazone which disperse the PMF by dissolving in the membrane and uncoupling ATP synthesis from electron transport (6). Similarly, some organic acids and their esters collapse the PMF by transporting protons into the cells (260). For instance, sorbic acid has been shown to accelerate the movement of protons in *Escherichia coli* from low pH media to the cell cytoplasm (260), while acetic acid was reported to neutralise the PMF in the cell (261).

As well as disrupting the cytoplasmic membrane and its PMF, biocides can interact with proteins and enzymes that are embedded in the cytoplasmic membrane. For instance, metals such as copper and silver are known to react with the thiol groups of proteins (607, 918, 1062), which are vital for the activity of many enzymes. Reaction with these important groups produces cell inhibition or cell inactivation. The phenolic biocide, hexachlorophene, was reported to inhibit the membrane-bound part of the electron

transport chain at low concentrations, whereas higher concentrations resulted in leakage of intracellular contents from *Bacillus megaterium* (312, 506).

1.3.3 Action on the cytoplasmic components

The cytoplasm contains a number of components that can be a target for biocide action. These include proteins, enzymes, ribosomes and nucleic acids. Many biocides are highly reactive chemicals and strongly interact with the bacterial cell components. For instance, aldehydes, alkylating and oxidizing agents all readily react with amino, carboxyl, sulphydryl and hydroxyl groups on proteins causing irreversible modification of the protein structure (676). Protein coagulation can also occur as result of biocide action. Compounds such as chlorhexidine, phenols and QACs have all been reported to cause such effect (676). Moreover, specific enzymes can also be targeted by biocides. It has been shown that triclosan targets the enoyl-acyl carrier protein reductase (FabI) in *E. coli* and *Mycobacterium smegmatis* (683, 685). In addition, primaquine, an acridine agent, was reported to block protein synthesis in *B. megaterium* (781), and proflavine was shown to inhibit the synthesis of polynucleotides by DNA polymerase in *E. coli* (509). Ribosomes can themselves be damaged by biocides. Although they might not be their primary targets, agents such as hydrogen peroxide, proflavine and *p*-chloromercuribenzoate can all damage ribosomes (698, 745, 1124).

Nucleic acids are also cytoplasmic components that can be targeted by biocides. The most obvious example are the acridines dyes which are nucleic acid stains that bind to the double stranded DNA by intercalation between adjacent bases on the same strand blocking replication and transcription (577). The triphenylmethane dye, crystal violet has been shown to interact with nucleic acids in *E. coli* (2). Alkylating agents such as ethylene oxide and formaldehyde, affect nucleic acids because of their interaction with the amino groups on the purine and pyrimidine bases (622). Modification of purine and pyrimidine bases is also seen in the action of the vapour-phase disinfectant, ozone (362), which decomposes in water to yield the hydroxyl and hydrogen peroxy radicals. These

are reactive species with oxidizing capacity, and ozone has even been reported to induce single-strand breaks in mammalian DNA (289, 543).

1.4 Biocide families

1.4.1 Phenols: Triclosan

Phenol and phenol containing product have a long history of use as antiseptics (459), and today enjoy a wide use as general disinfectants and as preservatives in a variety of products. They are however not allowed to be used where they can contaminate foods. Phenol, the parent compound, is effective against both Gram-positive and Gram-negative vegetative bacteria, but shows limited effect on spores and fast-acid bacteria. There are a large number of phenol derivatives which differ in their chemical reactivity depending on their structure. One of the most important in relation to biocides are the bis-phenols. These are hydroxyl-halogenated derivatives of two phenolic groups connected by various bridges, of which triclosan (2,4,4′-trichloro-2′-hydroxydiphenylether) (Figure 1.2) is the most wildly used.

Triclosan is a synthetic, non-ionic, broad spectrum antimicrobial agent which has mainly antibacterial but also some antifungal and antiviral properties (76, 502), as well as anti-inflammatory activity (45, 1099). Low concentration inhibits growth of mainly Gram-positive (including some mycobacteria) and Gram-negative bacteria. Much higher concentrations are bactericidal, although *Pseudomonas aeruginosa* and certain other bacteria are highly intrinsically resistant. In addition, yeast and moulds tend to be much less susceptible than *Staphylococcus aureus* to triclosan whereas bacterial spores are unaffected by it (912). The activity of triclosan compounds can be enhanced by formulation effects, and some reports have suggested that it may have anti-inflammatory activity as well as antimicrobial (45, 1099). Triclosan activity is not compromised by soaps, most surfactants, organic solvents, acids or alkalis but ethoxylated surfactants such as polysorbate 80 (Tween 80) entrap triclosan within micelles thus preventing its action (76). Although known for over 30 years, since its introduction into the health care

industry as a surgical scrub in 1972, the use of triclosan was confined to mostly health care settings for many years. The last decade however saw a rapid increase in the number of triclosan-containing products, these included skin cleaners, antibacterial hand rubs, dental products including toothpastes and mouth washes, deodorant soaps and other cosmetics (76, 502). Triclosan is also incorporated into fabrics and plastics, including toothbrush handles, cutting boards, children toys as well as surgical drapes and hospital over-the-bed table tops (961). Typically, triclosan is used at a concentration of 0.3% (w/w), although higher concentrations are also employed such as 2% recommended for skin decolonization of MRSA carriers (181).

Phenols induce progressive loss of intracellular constituents from treated bacteria and produce generalized membrane damage with intracellular coagulation occurring at higher concentrations (456).The initial reaction between a phenolic derivative and bacteria involves binding of the active phenol species to the cell surface. The compound then enters the cell either by passive diffusion (Gram-positives) or hydrophobic lipid bilayer pathway (Gram-negatives). The agent inhibits the cytoplasmic membrane-bound enzymes and causes its loss of the ability to act as a permeability barrier (126). Phenols denature protein structures by binding to amino acid residues, and the changes brought about in protein structure depend on concentration used. Small changes in protein structure can cause enzyme inhibition, whereas more significant changes in membrane proteins result in membrane damage and leakage of cell components (312, 844). If the protein is totally denatured, this results in coagulation of the proteins in the cytoplasm (626). Some lipid soluble phenols such as 2,4-dinitrophenol were reported to inhibit the membrane-bound part of the electron transport chain, and to dissipate the PMF (6).

The mode of action of triclosan has been well studied and it was initially thought that it is a membrane active, non-specific biocide affecting membrane structure and function (693, 861, 1095). However studies on *E. coli*, *S. aureus* and *P. aeruginosa* have shown that these organisms absorb triclosan by diffusion and that fatty acid composition of the cells affects their sensitivity to triclosan (693, 817). The major breakthrough in identifying the cellular target of triclosan came from studies on *E. coli* (412, 685), *P. aeruginosa* (441), *M. smegmatis* (683, 990), *M. tuberculosis* (990) and *S. aureus* (410). These studies all

showed that triclosan acts by blocking lipid biosynthesis by specifically inhibiting the NADH-dependent enoyl-acyl carrier protein reductase (FabI), or its homolog InhA in *M. smegmatis* (683) and *M. tuberculosis* (790, 990). The FabI is a major component of the fatty acid biosynthetic pathway in these bacteria, and also occur as part of a complex polypeptide in animal and fungi (682). Triclosan has also been shown to inhibit the enoyl-acyl carrier protein reductase of *Plasmodium falciparum* (57, 682, 1033). Although triclosan specifically targets the enoyl-acyl carrier protein reductase, in practice the agent is used at higher concentrations than those that cause the selective inhibition of fatty acid synthesis. Hence, the antimicrobial action of triclosan at in-use concentrations results from the non-specific damage to the cytoplasmic membrane, and the agent has been shown to induce potassium leakage from *S. aureus* at high concentrations (1031).

1.4.2 Quaternary Ammonium Compounds (QACs): Cetylpyridinium chloride (CPC)

Quaternary Ammonium Compounds (QACs) are organically substituted ammonium compounds in which the central nitrogen atom is joined to four organic radicals (Figure 1.1). QACs are used as cationic surface-active agents (surfactants) disinfectants, antiseptics and in drugs. Cationic surfactants are a class of chemicals that reduces surface tension at interfaces, and attaches to negatively changed surfaces, including micoorganisms. QACs were first recognized in the early 1900s and there were references to their use by Jacobs (488), and Jacobs *et al.* (489, 490). Nowadays they are recognized as one of the most useful antiseptics and disinfectants used in a number of clinical and veterinary procedures such as preparative disinfection of unbroken skin, application to mucous membranes, disinfection of noncritical surfaces (897) and disinfection of automatic calf feeders (722). In addition, QACs are also used as preservatives, sanitizers, in water treatment and other environmental purposes and are effective agents for hard-surface cleaning and deodorization (897).

One of the most useful QACs is benzalkonium chloride which at concentrations ranging from 0.005% to 0.2%, is used for the preoperative disinfection of unbroken skin, for application to mucous membranes, and for bladder and urethra irrigation (722).

Benzalkonium chloride (0.01%) is recognized as being suitable preservatives for inclusion in eyedrop preparations and is also widely used (at concentrations of 0.001–0.01%) in hard contact lens soaking solutions. The agent is also included in lozenges for the treatment of superficial mouth and throat infections, as well as in alcohol and acetone solutions at concentration of 0.2% for preoperative skin disinfection and for controlling algal growth in swimming pools.

The QAC examined in this study was cetylpyridium chloride (CPC) (Figure 1.2), a heterocyclic ammonium salts that comes as a white powder with a slight characteristic odour and is water soluble. Cetylpyridium chloride is used in a number of application including skin cleansing, treatment of wounds and burns, and a number of skin disorders. Solutions of 0.1-0.5% cetylpyridium chloride are used for skin disinfection and for antiseptic treatment of small wound surfaces (722). The agent is also incorporated in lozenges for the treatment of mouth and throat infections, as well as used for preservative in emulsions. Cetylpyridium chloride is incorporated in many cosmetic products (846) such as in hair preparations and in deodorants and in face and shaving lotions at low concentrations (0.05-0.1%).

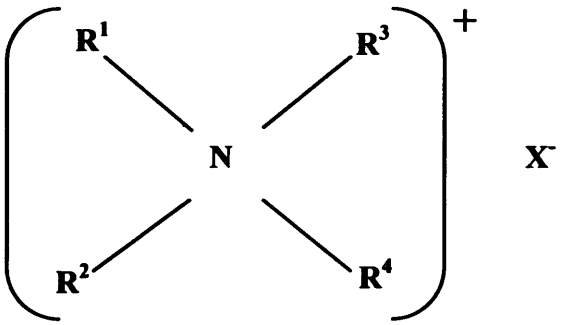


Figure 1.1. General structure of quaternary ammonium compounds.

R represent(s) alkyl or aryl substituents and X represents a halogen, such as bromide, iodide, or chloride

QACs are active against Gram-positive bacteria, with concentrations as low as 0.0005% being lethal. They are however less active against Gram-negative organisms (generally lethal at concentrations 0.0033%) especially *P. aeruginosa*, which tend to be highly resistant (221). It is thought that the high content of phospholipids and natural lipids in this organism increases its resistance (658). QACs are ineffective against mycobacteria (1038), presumably because of the lipid, waxy coat of these organisms, and show some antifungal properties although they are fungistatic rather then fungicidal (208). Viruses are more resistant than bacteria or fungi to the QACs (378). The spectrum of activity of QACs is concentration-dependent, whereby at low concentration they have a static effect, and at higher concentration they have a cidal effect.

QACs are membrane active agents affecting predominantly the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts (457). Salton *et al.* (935) proposed that micro-organisms exposure to cationic agents such as QACs leads to first the adsorption of the agent into the cell surface then its penetration and diffusion throughout the outer layers of the cell. This is then followed by the agent reacting with the cytoplasmic membrane (lipids or proteins) leading to membrane disorganization, leakage of intracellular low-molecular-weight material and degradation of proteins and nucleic acids leading to cell wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the cell (232).

In this context, QACs irreversibly bind to the phospholipids and proteins of the membrane, thereby impairing permeability. Compounds such as benzalkonium chloride have been shown to alter the hydrophobicity of Gram-negative bacteria (262, 263), and changes in the fatty acid composition of *P. aeruginosa* exposed to QACs have been reported (383). Additionally, QACs such as cetyltrimethylammonium bromide were reported to bind to nucleic acids and precipitate them, a property that is widely exploited in DNA preparations (227). These agents also induce leakage of intracellular components, which is indicative of membrane damage (217, 262, 263, 1041, 1049). As with chlorhexidine, low concentrations of QACs cause membrane damage and leakage of

cytoplasmic constituents, whereas higher concentrations cause cytoplasmic coagulation. Cetyltrimethylammonium bromide for instance, used at a bactericidal concentration, was shown to rupture the cell membrane (217). It was suggested that this compound targeted primarily the lipid components of the membrane and cell lysis was a secondary effect (345).

1.4.3 Biguanides: Chlorhexidine

A number of biguanides show antimicrobial activity, some of the most commonly used are chlorhexidine, alexidine and the polymeric forms notably polyhexamethylene biguanide (PHMB). Chlorhexidine is a 1,6-di(4-chlorophenyl-diguanido) hexane cationic bisbiguanide (Figure 1.2), and is available as dihydrochloride, diacetate and gluconate. Chlorhexidine was first synthesized in the 1950s and was found to have high-level antibacterial activity, low mammalian toxicity and a strong affinity for binding to the skin and mucous membranes (231). These properties amongst others such as its broad spectrum efficacy and low irritation made chlorhexidine one of the most widely used biocides in antiseptic products both in medical and veterinary settings (446, 919), in particular hand-washing and oral products. Chlorhexidine is used in concentrations ranging from 0.05% to 4% in washes and lotions (624) while chlorhexidine diacetate is typically used at a concentration of 2%. Chlorhexidine has also been formulated with other compounds such as ethanol and QACs for better effective use. It is used in oral conditions such as reducing dental plaques and treating gingivitis (195, 356), irrigating the bladder for some urinary infections, in gynaecology, cleaning wounds and burns, and catheterization procedures (1070). Although the main use of chlorhexidine is as an antiseptic, it is also used as disinfectant and a preservative (329).

Chlorhexidine has a wide spectrum of antibacterial activity against both Gram-negative and Gram-positive bacteria, although some may show resistance to this agent. For instance, strains of *Proteus* and *Providencia* spp., have been reported to be highly resistant to the biguanide (32, 480, 481, 900). Chlorhexidine is not lethal to acid-fast organisms, as low concentrations of the agent are mycobacteriostatic, but not generally mycobactericidal (894). Chlorhexidine is however, tuberculocidal in ethanolic solutions

(722). The biguanide is not considered sporicidal (898, 914), although it kills spores at 98-100^{0}C (722). The agent is active on protozoa (321), yeasts (439) and has some antiviral properties (792) although its not considered an effective antiviral. The activity of chlorhexidine is reduced in the presence of serum, blood and other organic matter, and as result of its cationic nature, activity is also reduced in presence of soaps and other anionic compounds.

The mode of action of biguanides especially that of chlorhexidine has been extensively studied (294, 295, 462, 463, 855, 915), however, most of these investigations were in relation to actions on non-sporulating bacteria (457). Chlorhexidine gluconate has been shown to be taken up very rapidly by bacteria (295) and fungal cells (438). At low concentrations of up to 200 μg/ml, chlorhexidine causes membrane damage, inhibits membrane enzymes and promotes leakage of cellular constituents. Leakage of cytoplasmic material generally increases with increasing concentrations up to a high concentration where cytoplasmic precipitation occurs. These high concentrations which lead to cytoplasmic coagulation also result in less leakage of cellular material and the bactericidal effect is seen. Low concentrations of chlorhexidine have bacteriostatic activity whereas high concentrations are rapidly bactericidal (231).

Evidence of the above has been collected from a number of studies and reports. For example, the hydrophobicity of Gram-negative bacteria was altered when subjected to chlorhexidine (262, 263, 294, 500), and the agent was shown to damage the cell wall and outer membrane of these bacteria and to promote its own uptake to reach its target(s) at the cell cytoplasmic membrane, where it interacts with the anionic lipids (900, 935), and cytoplasm (343). Chlorhexidine was reported to induce potassium leakage from baker's yeast and affect the ultrastructure of budding *Candida albicans* with loss of cytoplasmic constituents (84). Chlorhexidine was claimed by Harold *et al.* (401) to be an inhibitor of both membrane-bound and soluble ATPase as well as of net potassium ions uptake in *Enterococcus faecalis*. However, this may not be the primary target for the agent as only high biguanide concentrations inhibit membrane-bound ATPase (162).

Barrett-Bee *et al.* (48) studied the membrane distabilising action of chlorhexidine in a number of bacterial species including *E. coli*, *S. aureus*, *Morganella morganii* and *P. aeruginosa*. They reported that the agent caused inhibition of oxygen utilisation in the bacteria that was related to fall in cellular ATP levels, although this was not responsible for the bactericidal effect of the compound. Investigators also noted effect(s) on the outer membrane of Gram-negative bacteria which allowed the release of periplasmic enzymes. Although the inner membrane was not ruptured the agent caused it to be functionally breached, which was coupled with inhibition of the active uptake of small molecules, but not related to cellular ATP levels.

1.4.4 Aldehydes: *Ortho*-phthalaldehyde (OPA)

Aldehydes are wide spectrum activity biocides, of which glutaraldehyde (GTA) and formaldehyde are the most important as disinfectants. GTA is a saturated five-carbon dialdehyde with an empirical formula of $C_5H_8O_2$. GTA is more stable at acid than alkaline pH, and solutions at pH 8 and above generally lose activity within 4 weeks. For this reason, GTA is usually obtained commercially as a 50% solution of acidic pH, and is used in disinfection as a 2% solution that is activated (made alkaline) before use. GTA have been recommended as disinfectant and sterilant, in particular for low-temperature disinfection and sterilization of endoscopes, arthroscopes, laparoscopes, and surgical equipment and as a fixative in electron microscopy (620, 897). It has also been used in the veterinary field for the disinfection of utensils and of premises (919). GTA has many advantages as a biocide, as it has broad spectrum of activity with rapid microbial action, and it is non-corrosive to metals, rubber and lenses (722). However, there is concern over its toxicity, and potential mutagenic and carcinogenic effects of GTA have been reported (464) as well as skin and eye irritation and respiratory disorders (904). This, along with the appearance of GTA-resistant *M. chelonae,* lead to the introduction of a new GTA-alternative aldehyde, *ortho*-phthalaldehyde (see below) (1119). GTA has a wide spectrum of activity and was shown to be effective against both sporulating and non sporulating bacteria, fungi, and have a potent mycobactericidal (1119), sporicidal (357), and viricidal activity (284).

Formaldehyde is the other important disinfectant in the aldehyde group, and is used both in liquid or vapour forms. The formaldehyde solution (formalin) is typically 34-38% w/w CH_2O and contains methyl alcohol to delay polymerization (722). Liquid formaldehyde solutions have been used as viricidal agent in the production of vaccines such as polio, treatment of warts, as preservative in hair shampoos, as an antiseptic mouthwash, as disinfectant of membranes in dialysis equipment, and as a detergent in RNA gel electrophoresis, preventing RNA from forming secondary structures (722). Formaldehyde vapor has been employed in the disinfection of sealed rooms, hospital bedding and blankets, and sterilization of heat-sensitive medical materials. Formaldehyde was first reported as disinfectant in 1892 and it is microbicidal and lethal against bacteria and their spores, fungi and viruses. However its activity is influenced by organic load and especially in vapor form its effectiveness is dependent on concentration, temperature and relative humidity (722).

As mentioned above, concerns over the toxicity and development of resistance to GTA, lead to the introduction of a new-GTA alternative, *ortho*-phthalaldehyde (OPA) (1119). *Ortho*-phthalaldehyde is itself an "old" molecule, but recently it has been examined in a new antimicrobial context. *Ortho*-phthalaldehyde is an aromatic dialdehyde (Figure 1.2) that comes in the form of a yellow crystals or powder, and is used at a concentration of 0.55% (w/v) (310). Its activity has been studied in both Gram-positive and Gram-negative bacteria (311, 1120, 1121) and in mycobacteria (1120), and it is claimed to have potent bactericidal, sporicidal and viricidal activity and has been suggested as a replacement for glutaraldehyde in endoscope disinfection (11, 363, 1119, 1120). To date little is known about the mechanism of action of *ortho*-phthalaldehyde, however early evidence suggest that its action is similar to that of GTA (1118).

Aldehydes are reactive molecules and are able to react with residues on both proteins and nucleic acids by alkylation, leading to irreversible chemical modification which results in the inhibition of metabolism and cell division. Among the chemical groups that aldehydes are able to react with are amino, carboxyl, thiol, hydroxyl, imino, and amide substituents (577). Cross-linking of proteins is also observed and usually involves

multiple interactions between chemical groups leading to aggregation (495, 886). Formaldehyde was reported to act on proteins by the same above process (495), and on nucleic acids by alkylation, for example alkylation of the amino groups on purine and pyrimidine bases (622). The mode of action of GTA is similar to that of formaldehyde, and is thought to involve strong association with the outer layers of bacterial cells (120), and it acts by intermolecular cross-linking of bacterial proteins, such as lipoproteins (986). Although GTA does not damage bacterial spores DNA, it eliminates their ability to germinate (912). The new GTA-substitute, *ortho*-phthalaldehyde, was reported to interact with the amino acids, proteins, and microorganisms, although not as affectively as GTA. It was shown to cause less cross-linkage, and its high activity against mycobacteria was a result of its lipophilic nature, which aids its uptake thought the cell wall (986).

1.4.5 Other biocide families

1.4.5.1 Alcohols

Alcohols have long been used as biocides, and are generally rapidly bactericidal (728), in some cases including fast-acid bacteria (201), but they are not sporicidal even at high concentration (901). They have low activity against some viruses (1080), but are viricidal towards others (1086). Alcohols are generally considered non-specific antimicrobial agents because of their many toxic effects. Their mode of action appears to involve a number of effects including protein coagulation and denaturation, disruption of the cytoplasmic membrane and cell lysis (126, 296, 966). They also interfere with the cellular metabolism causing inhibition of DNA, RNA, protein and peptidoglycan syntheses along with other effects such as inhibition of the enzymes involved in glycolysis, fatty acid and phospholipid syntheses and effects on solute uptake (913, 966).

1.4.5.2 Halogens

The two most important microbial halogens are iodine and chlorine compounds. Iodine and its derivatives are mainly used for antisepsis (658) and are considered efficient

microbial agents able to rapidly kill bacteria and their spores, moulds, yeast and viruses (333, 531, 658, 901, 1038). Iodine interacts with the thiol groups of enzymes and proteins (456, 913), which are important determinants of protein structure and function, leading to metabolic inhibition of the cell (185, 359, 1061). Moreover, there is evidence that this agent acts by interacting with the double bonds of the phospholipids causing damage to the cell wall and leading to loss of intracellular material (126).

Chlorine compounds are widely used as sanitizing agents in the food industry and as disinfectants, of which hypochlorite is one of most commonly utilised (722). Hypochlorites are considered wide spectrum, antibacterial agents effective against non-sporulating bacteria but with low activity against mycobacteria (201). They are active against viruses (725) and are considered among the most potent sporicidal agents (177, 178, 532). Chlorine compounds act on multi-targets in the cell including cell wall and amino groups on proteins leading to the metabolic inhibition of the cell (456, 909), as well as deleterious effects on DNA synthesis resulting from the formation of chlorinated derivatives of nucleotide bases (676, 681).

1.4.5.3 Peroxygens

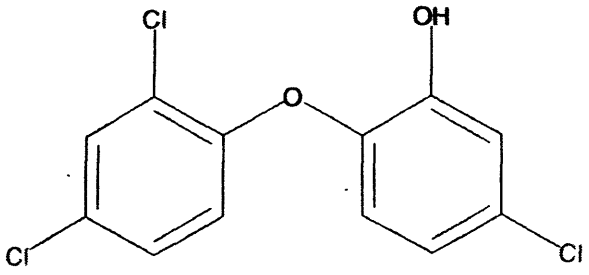
The most important peroxygens used as biocides are hydrogen peroxide (H_2O_2), peracetic acid (CH_3COOOH) and ozone (O_3) (83, 722, 836, 1130). They possess disinfectant and antiseptic properties and are effective against a wide range of organisms including bacteria, yeast, fungi, viruses, and spores (37, 126, 387, 477, 878, 898, 902, 911, 994, 1130). Peroxygens are powerful oxidants, and it is through the formation of the hydroxyl radicals that these agents exert their antimicrobial effect. Being highly active, the hydroxyl radicals oxidise thiol groups in enzymes and proteins (232, 341, 909) as well as other chemical groups represented within a whole range of membrane-bound and intracellular enzymes. They can also attack other components of the cell wall, membrane and cytoplasm, including membrane lipids, DNA, and RNA, causing cell destruction (289, 362, 475, 535, 543).

1.4.5.4 Heavy metals derivatives

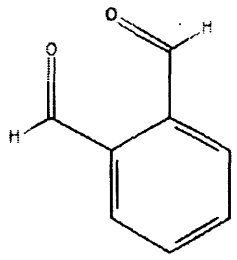
Among the heavy metals derivatives, copper, silver and mercury compounds are the most commonly used. They are used as antimicrobial agents as well as in the activation and increase efficiency of other drugs and biocides (676, 1189). Heavy metals such as copper and silver ions were reported to cause structural changes in the cell envelope and induce cytoplasmic protein coagulation. They also react with the chemical groups on proteins, enzymes and DNA (918), leading to cell inhibition and inactivation (607, 918, 1062).

1.4.5.5 Antimicrobial dyes

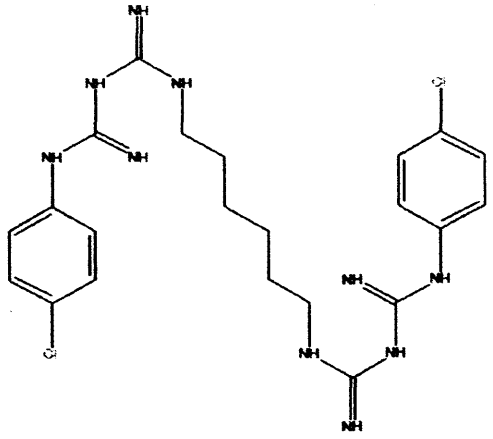
Antimicrobial dyes have antimicrobial use of and acridines, triphenylmethane group and quinines are the most commonly employed. They are mainly used as antiseptics and act by combining with several sites on or in the bacterial cell, including DNA, and RNA (352, 984). They inhibit gene replication and expression by blocking DNA, RNA, and protein synthesis (2, 171, 750, 967, 1153).



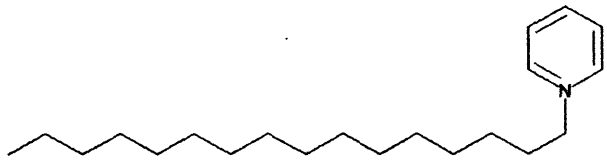
A



B



C



D

Firure 1.2. Chemical structures of the biocides used in this study.

A; triclosan, B; *ortho*-phthalaldehyde, C; chlorhexidine, and D; cetylpyridium chloride.

1.5 Biocide resistance in bacteria

Resistance to biocides has been widely studied in bacteria (676, 913), as well as, to a lesser extent, in fungi and some protozoa (537, 917, 1078). Reports of bacterial resistance to biocides included high and low level resistance to triclosan in *S. aureus* (40, 190, 410, 412, 946, 1030, 1031), low level resistance to QACs and chlorhexidine in *S. aureus*, *P. aeruginosa* and *P. stutzeri* (40, 501, 690, 921, 1030, 1049), resistance to diamidines in *S. aureus* (613, 1054), and resistance to glutaraldehyde in *M. chelonae* (656). The term "bacterial resistance" is often used loosely in the context of biocides. For antibiotics, resistance in a bacterium is usually referred to in a clinical context, where a bacterium became able to withstand an antibiotic concentration to which it was sensitive, hence making the agent's concentration ineffective therapeutically. By contrast, much of the work investigating biocide resistance is laboratory-based, and most of the resistance reported has little clinical significance as the levels of resistance recorded are to biocide concentrations well below those used in hospital, domestic or industrial practice (152, 896).

It is also the case that resistance is generally determined from MIC values which describe the biocide growth inhibitory effect but do not necessarily reflect its effectiveness at killing the organism. As biocides efficiency and benefits are dependent for the most part on producing effects that cause rapid kill, resistance determined from MIC results has little relevance in assessing whether the MIC values are correlated to reduced kill or product failure. Nevertheless, bacterial species, such as *P. aeruginosa* and *M. tuberculosis*, are able to survive "in use" biocide concentrations, and Russell (895-897, 899, 905, 906) described several biocides including QACs, bis-biguanides, diamidines, bis-phenol, and acridines, to which resistance may be a problem. Bacterial "reduced susceptibility" to biocides, which is the more correct term rather than biocide "resistance", can arise in a number of mechanisms. Some of these are intrinsic, which are inherent features of the organism, and others are acquired.

1.5.1 Intrinsic biocide resistance

Intrinsic resistance is a natural property of an organism and is shown by bacterial spores, mycobacteria, and some Gram-negative bacteria (907). Some bacteria withstand antimicrobials because their innate biochemical makeup renders them intrinsically less susceptible. Resistance to biocides is mostly due to intrinsic cellular mechanisms (1141), and this inherent biocide resistance is likely to be of greater significant in Gram-negative bacteria (910). This form of resistance is usually associated with cellular impermeability, efflux, and degradation or inactivation of the biocide. Intrinsic resistance is usually a result of contribution from many if not all of the above mechanisms, whereby the protein-lined pores of the outer membrane restrict the access of hydrophobic and large hydrophilic molecules to the vulnerable cytoplasmic membrane. The complex outer membrane structure and its rigid lipid bed slow down the penetration of hydrophobic molecules, while a strong efflux system ensures that lipophilic biocides that do penetrate the envelope are pumped out of the cell. Those agents that succeed in passing these mechanisms can be inactivated or degraded by specialized pathways.

1.5.1.1 Cellular impermeability

Intrinsic biocide resistance is usually shown as a reduced uptake of the agent which occurs as a result of impermeability barriers (913) in bacterial spores, mycobacteria, Gram-negative bacteria, and vancomycin-resistant *S. aureus* strains (899). Vancomycin resistance arises as result of mutation and cell-wall thickening and alteration in peptidoglycan (440). Biocides interact with microorganisms initially at the cell surface; hence intrinsic resistance is significantly influenced by the composition and components of the cells outer surfaces. Bacteria differ considerably in their response to biocides. The most resistant are undoubtedly bacterial spores, followed by mycobacteria, Gram-negative bacteria, and then Gram-positive bacteria. There are however numerous exceptions to this classification (897) as well as wide differences in susceptibility within the above groups of organisms (676).

Many of the differences in biocide susceptibility between the different microbial groups can be explained on the basis of the structure, composition and components of the cells surfaces. In spores their outer and inner coats (composed of alkali-resistant S-S bounds and alkali-soluble acidic polypeptides respectively), their cortex, made up of the peptidoglycan, limit the uptake of biocides (676). It is however also known that spores have other intrinsic mechanism of biocide resistance in addition to their cellular impermeability. For instance, spores ($\alpha\beta$) lacking the major DNA protective α/β-type small, acid-soluble spore protein, were shown to be more susceptible to peracetic acid and formaldehyde (622).

In terms of their biocide response, mycobacteria occupy an intermediate position between bacterial spores and other bacteria (894). The main reason for their biocide resistance is their lipid-rich waxy cell wall, which limits the uptake of many biocides (310, 894). Studies on GTA-resistant *M. chelonae* strains suggested that the arabinogalactan/arabinomannan component of the cell wall is associated with resistance to the aldehyde. It is however interesting that *ortho*-phthalaldehyde, a cyclic dialdehyde is shown to be effective against the GTA-resistant strains (986, 1121). The mycobacteria cell wall structure and its action as a permeability barrier are discussed in more detail in section 2.1.7.

Gram-negative bacteria, especially *P. aeruginosa*, *Proteus* spp., *Providencia* spp., and *Serratia marcescens*, generally show reduced susceptibility to biocides compared with Gram-positives (913). A major reason for this reduced susceptibility is that the Gram-negative bacterial cell wall significantly limits the uptake of these agents. The outer membrane of Gram-negative bacteria acts as a permeability barrier due to its narrow porin channels, which limit the penetration of hydrophobic molecules, and to the low fluidity of the lipopolysaccharides leaflets, which slow down the diffusion of lipophilic compounds into the cells.

It was thought that the antimicrobial agents that are less active on Gram-negative bacteria compared with Gram-positives, acted by inducing metabolic or structural changes in the

cytoplasmic membrane (458). The cytoplasmic membranes of both Gram-negative and Gram-positive bacteria were subsequently shown to be equally sensitive to the action of these agents (394). Hamilton (394) suggested that the layer of the Gram-negative cell envelope external to the cytoplasmic membrane may either constitute a non-absorbing barrier or may absorb and retain the agents, thus protecting the underlying sensitive membrane. Unlike in Gram-positives, the Gram-negative cell envelope is a complex and multilayered structure. In addition to the typical inner cytoplasmic membrane, Gram-negatives have an additional outer membrane composed of lipopolysaccharides, phospholipids, and proteins. Sandwiched between these two membranes is the periplasm, some 10-25 nm in depth (360), and containing the peptidoglycan layer and enzymes, including β-lactamases, ribonucleases and phophatases, suspended in a highly hydrated polysaccharide gel (1017).

The peptidoglycan consists of glycan chains cross-linked by peptides. The sugar component of peptidoglycan consists of alternating residues of β-(1,4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. Attached to the *N*-acetylmuramic acid is a peptide chain of three to five amino acids. The peptide chain can be cross-linked to the peptide chain of another strand forming a 3D mesh-like layer which provides strength and rigidity to the cell wall. In Gram-negatives the peptidoglycan is attached to the outer membrane via lipoproteins (1140), and it is less substantial than in Gram-positives, typically 3-5 nm thick (360). Although not considered a major factor associated with cell permeability, Denyer and Maillard (233) suggested that peptidoglycan plays an indirect role by holding together the outer membrane of the cell.

The structure and composition of the outer membrane is different from that of the cytoplasmic membrane in that it contains less phospholipids (located in the inner surface), fewer proteins and has a unique component, lipopolysaccharides (located on the outer leaflet of the membrane) (1017). Lipopolysaccharides are characteristics of the Gram-negative outer membrane and consist of three covalently-linked regions (233). The first is a phosphorylated glucosamine dissacharide unit attached to a number of fatty acids, called lipid A. The second region is the core polysaccharide, a complex

oligosaccharide of about 10 sugar residues, linked to lipid A by 2-keto-3-deoxyoctonate. The final portion is the O-side chain which is joined to the core polysaccharide and is composed of repeating subunits of oligosaccharides. The lipopolysaccharides chains have high negative charge that provides a polyanionic external surface, partially neutralised by divalent cations (Mg^{2+} and Ca^{2+}). These non-covalent cross-links formed by the cations give structural strength and integrity to the lipopolysaccharides chains in the outer surface, as well as limiting the access of hydrophobic molecules into the bacterial cell (398).

The Gram-negative outer membrane also contains porin proteins which form channels across the lipid bilayer. These channels function in the transport of hydrophilic low molecular weight substances (including some hydrophilic drugs) into the cell. The size of the solute and its chemical composition as well as the size of the channel are important determinants to whether the molecule is able to diffuse thought the outer membrane into the cell. In *E. coli*, porin proteins OmpF and OmpC, which allow the non-specific diffusion of hydrophilic molecules, are folded in such a way as to produce a barrel-shaped, water-filled channel across the outer membrane (763). The channels do not have a constant cross-section diameter, and are wide at the entrances and exits but have a narrow central section of only 0.7 X 1.1 nm (764). This excludes molecules with molecular weight higher than 600 Da from passing across the outer membrane. Although it is possible for molecules with higher molecular weight than 600 Da, and which are long, flexible and hydrophilic to slowly cross the channels (764).

P. aeruginosa is able to intrinsically restrict the uptake of biocides due to the high Mg^{2+} content in its outer membrane, which aids in producing strong lipopolysaccharides-lipopolysaccharides links (676). The tight packaging of the six fatty acids in lipid A molecules in *E. coli* was suggested to play a role in the rigidity of the interior of the lipopolysaccharides manolayer, resulting in poor diffusion of hydrophobic molecules (762). Tamaki and Matsuhashi (1047) demonstrated that *E. coli* rough mutants, with extensive lipopolysaccharides effects were unusually sensitive to the hydrophobic antibiotic novobiocin and hypersensitive to the enzyme lysosyme. From this observation

the authors concluded that the lipopolysaccharides on the cell surface of the cell prevent penetration of lysozyme and certain low-molecular-weight drugs (1047). A contributory factor in the resistance of *Proteus* species to chlorhexidine and other cationic biocides is the presence of a less acidic type of outer membrane lipopolysaccharides (913). *Burkholderia cepacia* has been reported to be resistant to chlorhexidine (784) and triclosan (502). The unusual high concentration of phosphor-linked arabinose in the lipopolysaccharides of the organism decreased the affinity of the outer membrane to cationic agents (198).

Mutants producing lipopolysaccharides with polysaccharide chains of different lengths are available in *Salmonella,* and provide a unique opportunity for examining how alterations in the structure of this integral component affect the postulated barrier properties of the outer membrane. *Salmonella typhimurium* "deep rough" mutants whose lipopolysaccharides lack most of the saccharide chains are highly sensitive towards some antibiotics, crystal violet, malachite green and phenol (883, 954). In the same organism, the "*omp* mutants", which exhibit a normal wild-type lipopolysaccharides composition but have a reduced level of outer membrane proteins, were shown to be sensitive to crystal violet and deoxycholate (15). These mutants also allowed rapid penetration of a number of hydrophobic antibiotics (761).

1.5.1.2 Efflux pumps

Studies have shown that broad specificity efflux pumps, named multidrug efflux pumps, also contribute to the intrinsic resistance of Gram-negative bacteria to a variety of agents including dyes, detergents, and antibiotics (601, 603, 758, 760, 765, 835). These pumps are chromosomally encoded and induced through sublethal exposure to compounds including small hydrophilic antibiotics and agents such as QACs, pine oil, and salicylate (704). Hence, bacteria exposed to sublethal concentrations of some biocides might induce multidrug resistance for as long as the pump is actively expressed. There are five major superfamilies of efflux pumps, each family contains pumps that are specific for single

agents together with pumps that are responsible for multidrug efflux (845). These superfamilies are:

- The small multidrug resistance family (SMR) (170), now described as part of a larger drug/metabolite transporter superfamily (DMT) (483).
- The major facilitator superfamily (MFS) (788).
- The multidrug and toxic compound extrusion family (MATE) (110).
- The resistance-nodulation-division family (RND) (758, 1193).
- The ATP-binding cassette family (ABC) (1091).

These efflux systems can further be divided into two classes based on the mechanism they use to pump agents out of the cell. Those that use transmembrane electrochemical gradient of protons or sodium ions to actively efflux agents from the cell are referred to as secondary drug transporters, and include the MDR, RND, SMR, and MATE systems (845). The ATP-binding cassette family (ABC) belongs to the second class and uses energy of ATP hydrolysis to pump agents out of the cell (845). Structure of the main types of efflux systems in bacteria are shown in Figure 1.3.

Two efflux pumps are well-established in relation to conferring intrinsic resistance to biocides in Gram-negative bacteria. These are the MexAB-OprM system in *P. aeruginosa* (Figure 1.3) and the AcrAB-TolC system in *E. coli*. Both pumps are of the RND-type and have a three-component organization (1193) which includes a transporter located in the inner membrane, an outer membrane channel that functions with the transporter and a periplasmic accessory protein (Figure 1.3). In *E. coli*, the AcrAB-TolC system is encoded by the *acr* genes, and is composed of AcrB which spans the inner membrane of the cell, an accessory periplasmic protein AcrA, which is also anchored in the inner membrane, and an outer membrane protein, TolC. The pump is under the

regulation of the *mar* (multiple antibiotic resistance) operon, and acts as a transporter for tetracycline, ciprofloxacin, fluoroquinolone, β-lactams, and novobiocin as well as ethidium bromide, acriflavine, phenylethylalcohol, sodium dodecyl sulfate, and deoxycholate (636-638, 744, 780). Activation and up-regulation of the AcrAB-TolC efflux pump was seen in *E. coli* mutants which over-expressed the Mar protein. These mutants expressed increased resistance to antibiotics, cyclohexane, pine oil, bile salts, and disinfectants such as triclosan, QACs and chlorhexidine (601, 719).

The MexAB-OprM system in *P. aeruginosa* is the homologue of the AcrAB-TolC system in *E. coli*. The MexAB-OprM pump which is constitutively expressed, and has a normal physiological function of exporting the siderophore pyoverdine into the surrounding medium, was shown to be able to pump out a wide range of structurally unrelated antibiotics (759). The system was also shown to be capable of transporting triclosan from *P. aeruginosa* cells (166, 961). In *P. aeruginosa,* in addition to the MexAB-OprM pump, other Mex systems (MexCD, and MexEF), which transport a variety of agents, including tetracycline, ciprofloxacin, fluoroquinolone, β-lactams, and fusidic acid, also exist. Schweizer (960) suggested that for *P. aeruginosa* the presence of the Mex systems coupled with the narrow porin channels in its outer membrane, which restricts the diffusion of antimicrobial agents into the cell are responsible for the very high intrinsic resistance of this species to antimicrobial agents compared to other Gram-negative bacteria. *P. aeruginosa* RND-type systems have also been identified in a number of Gram-negative pathogens including *B. cenocepacia*, *B. pseudomallei*, *Stenotrophomonas maltophilia*, and in the non pathogen *P. putida* (959). Similarly, *E. coli acr*-like systems have been found in other species of *Enterobacteriaceae* such as *Salmonella* spp. (760, 765, 1028)

1.5.1.3 Biocide inactivation/degradation

In addition to impaired uptake and increased efflux, micro-organisms show intrinsic resistance though inactivation or degradation of biocides. Nishihara *et al.* (769) reported a *P. fluorescens* strain TN4 isolated from a sewage treatment plant, that was highly

resistant to and able to degrade a number of QACs and detergents including didecyldimethylammonium chloride, and alkyltrimethyl- and alkylbenzyldimethyl-ammonium salts. El-Sayed *et al.* (769) isolated two phenol tolerant bacterial strains, *B. cepacia* PW3 and *P. aeruginosa* AT2, which demonstrated high biodegradation activity against this agent. Enzymatic degradation of triclosan has been demonstrated in two soil bacteria, *P. putida* TriRY and *Alcaligenes xylosoxidans* subsp. *denitrificans* TR1, which were shown to grow on medium containing 1% triclosan (689). Triclosan degradation has also been shown in *Sphingomonas* sp. strain RD1, where loss of the ability to mineralize triclosan resulted in susceptibility to this biocide (510).

Some intrinsically formaldehyde resistant *Pseudomonas* species which expressed an aldehyde dehydrogenase were described by Russell and Chopra (913). Chlorhexidine resistant *Achromobacter xylosoxidans* isolated from an ultrasonic hand washer was reported to be able to degrade the biguanide (913). Degradation of chlorhexidine was also described by Kido *et al.*(541) in *Pseudomonas* and *Flavobacterium* species, which were able to utilize the biguanide as the sole nitrogen source for growth. Similarly, a study by Uyeda *et al.* (1082) showed that *Pseudomonas* species and *S. marcescens* were both able to degrade chlorhexidine. Resistance to the ester *para*-hydroxybenzoic acid, a widely used preservative in food and cosmetics, was attributed to hydrolysis by an esterase in both *B. cepacia* and *Enterobacter cloacae* (151).

1.5.2 Acquired biocide resistance

Acquired resistance arises via mutation, amplification of an endogenous chromosomal gene, the acquisition of genetic elements (plasmids, transposons, or transformation), and through adaptive phenotypic changes (833, 910). This form of resistance can be either plasmid-mediated or non-plasmid mediated.

1.5.2.1 Non-plasmid mediated resistance

Acquired non-plasmid mediated bacterial resistance to biocides can occur by mutation in the biocide target site, acquisition of genetic elements other than plasmids, and phenotypic adaptation, and may involve changes in the membranes compositions, over expression of efflux systems, and biocide inactivation.

Acquired biocide resistance via phenotypic adaptation may result when bacteria are "trained" to grow in gradually increasing concentrations of a biocide (907). Although this type of resistance mechanism has been reported, it is considered unlikely to play an important role in long-term tolerance of bacteria to biocides (162). Tattawasart *et al.* (1049) reported stable chlorhexidine and QACs resistance in *P. stutzeri* after being exposed to gradually increasing concentrations of either antibacterial agent. Changes in the outer membrane proteins of the resistant strains were observed (1048). Loughkin *et al.* (623) trained *P. aeruginosa* strains to tolerate the QAC disinfectant benzalkonium chloride, by growing them in increasing concentrations of the agent. Two strains showed stable increase in resistance to the agent as well as to other QACs and some antibiotics. The strains showed alterations in outer membrane proteins, uptake of benzalkonium chloride, cell surface charge and hydrophobicity, and fatty acid content of the cytoplasmic membrane. Guerin-Mechin *et al.* (383, 384) and Mechin (690) reported changes in inner and outer membrane fatty acid composition consistent with changes in lipopolysaccharides and in the hydrophobicity of the membrane cores in QACs-adapted *P. aeruginosa* cells.

Overexpression of efflux systems due to mutations or induction after exposure to antimicrobial agents is also a common mechanism in acquired biocide resistance. There are a number of agents which are substrates for efflux pumps including biocides such as triclosan (964) but not inducers of their expression. Prolonged exposure to sublethal concentrations of such agents would select for mutants which constitutively express efflux pumps. Chuanchuen *et al.* (166) exposed a susceptible *P. aeruginosa* mutant population, in which the *mexAB* was deleted, to triclosan. They reported that this

exposure selected a multi-drug-resistant strain that hyperexpressed the MexCD-OprJ efflux system due to mutations in its regulatory gene, *nfxB*. In *E. coli*, overexpression of the multidrug efflux pump locus *acrAB*, or of *marA* or *soxS*, both encoding positive regulators of *acrAB*, conferred triclosan resistance (684). Similar results were observed in relation to resistance to disinfectant pine oil, where overexpressed the *marA* gene conferred resistance to pine oil and multiple antibiotics (tetracycline, ampicillin, chloramphenicol, and nalidixic acid) (719).

Chromosomal alterations and changes in biocide target site can also lead to decreased susceptibility. An example is triclosan, which has been shown to target the enoyl-acyl carrier protein reductase of fatty acid biosynthesis (FabI) in a number of bacterial species including *E. coli* (685), *S. aureus* (410), *P. aeruginosa* (441), *Haemophilus influenzae* (657), *B. subtilis* (411), and in both *M. smegmatis* (683) and *M. tuberculosis* (790) (where is it called InhA). Exposure of *E. coli* to sublethal concentrations of triclosan has been shown to select, at relatively high frequency, clonal mutations that are either modified in the *fabI* gene, encoding the enzyme, or where the gene has been repressed or deleted (342). In either instance, the susceptibility is reduced, giving rise to a series of mutants with increasing levels of resistance. Hyperexpression of a modified *fabI* gene product was also observed in triclosan-resistant *S. aureus* (280). Moreover, It has been shown that mutations in the *inhA* gene of *M. tuberculosis* and *M. smegmatis* and the *fabI* gene of *S. aureus* also provide triclosan resistance (410, 683, 790).

1.5.2.2 Plasmid mediated resistance

The first evidence that plasmids can encode reduced susceptibility to biocide agents involved heavy metals. Although a limited number of heavy metals have been employed as biocides, they are by definition biocidal (834). Bacterial resistance to mercury is mainly plasmid-borne, inducible and may be transferred by conjugation or transduction (910). Resistance to mercury has been widely reported often in strains multiply resistant to other biocides or antibiotics (833), and is determined by the *mer* genes which are mainly plasmid-encoded, although chromosomal *mer* genes have been reported, often on

highly mobile transposable elements (705, 712). These genes include *merA*-encoded mercury reducatse (833), *merB* encoding an organomercuricallyase (833), the regulatory gene, *merR*, the transport genes, *merT* and *merP*, and genes *merC*, and *merD* (705, 712).

Resistance to silver is of importance as the agent is widely used as a biocide (386). Silver salts such as silver nitrate and silver sulphadiazine are particularly important topical antimicrobials (722) and resistance to these agents has been reported in clinical isolates (385, 386, 536, 833). Plasmid-mediated resistance to silver has been reported in *P. stutzeri* (389), *Enterobacteriaceae* (985, 1073) and *Citrobacter* species (1073). Gupta *et al.* (385) reported the molecular basis of silver resistance in clinical isolates of *Salmonella*. They demonstrated that the resistance is a result of two plasmid-encoded efflux determinants, *silP* and *silCBA*. Another gene, *silE*, encoding a periplasmic silver binding protein, has also been described and is proposed to sequester silver, compromising its access to silver sensitive targets in the cell (385).

Formaldehyde resistance has been identified among several members of the family *Enterobacteriaceae* and *Pseudomonas* species (392, 419, 521, 1157). In a number of *Enterobacteriaceae* species including strains of *E. coli*, formaldehyde resistance was shown to be plasmid-mediated and self-transmissible (521, 1157). A cloned DNA fragment containing the formaldehyde resistance determined from *E. coli* was shown to hybridize with DNA from formaldehyde-resistant *S. marcescens*, *E. cloacae*, *Citrobacter freundii* and *Klebsiella pneumoniae* strains (1157). In formaldehyde-resistant *E. coli* VU3695, the resistance is encoded by the *adhC* gene located within the large self-transmissible plasmid pVU3695 (569). The *adhC* gene encodes a glutathione-dependent formaldehyde dehydrogenase (1081) for which primary function is the metabolism of endogenous formaldehyde, although it is also involved in microbial resistance to formaldehyde when used as disinfectant (522, 948). Kaulfers (525) and Kaulfers *et al.* (524) demonstrated that the plasmid-mediated formaldehyde resistance in *E. coli* and *S. marcescens* was associated with changes in the outer membrane proteins. Other plasmids able to cause changes in the outer membrane proteins of *E. coli* include plasmid R124

which was shown to alter the surface of the *E. coli* cells, notably the OmpF outer membrane protein, rendering the cells more resistant to cetrimide and other agents (889).

The genetic aspects of plasmid-mediated biocide resistance have been extensively studied in staphylococci (947). *S. aureus* strains carry a number of plasmids many of which encode antibiotic resistance. Resistance to acridines, ethidium bromide, QACs, and propamidine isethionate is mediated by a common determinant on a group of structurally related plasmids. Many of these plasmids also carry transposon Tn*4001* encoding resistance to gentamicin, tobramycin and kanamycin, as well as the dihyrofolate reductase inhibitor, trimethoprim (910). Numerous studies reported increased MICs of some biocides in *S. aureus* strains that possessed a plasmid carrying genes encoding resistance to gentamicin (121, 184, 346-348, 632, 633, 850, 988, 1068, 1069). It is now known that reduced susceptibility to ethidium bromide, acriflavine, QACs such as cetrimide and benzalkonium chloride, and diamidines such as propamide isethionate is mediated by this group of structurally related plasmids carrying the *qac* genes and encoding QAC efflux pumps (59, 63, 161, 614, 634, 713, 800, 870, 1054)

Both *qacA* and *qacB* genes encode proton-dependent export proteins which show significant homology to other energy-dependent transporters such as the tetracycline exporters found on various tetracycline-resistant bacteria (887). The *qacA* gene is found predominantly on the pSK1 family of multiresistance plasmids, and its expression is governed by QacR, a repressor of *qacA* (375). The *qacB* gene is found on many β-lactamase and heavy metal resistance plasmids such as pSK23 (887, 908). Although coding for similar proteins as *qacA, qacB* is more specific and relates only to intercalating dyes and QACs (798, 800, 801). The *qacC* and *qacD* genes encode the same polypeptide and have identical phenotypes conferring resistance to ethidium bromide and some QACs and are typically found on the plasmids pSK89 and pSK41 (342). The origins of the staphylococcal *qac* genes are unclear, but it has been suggested that they evolved long before the introduction and use of topical antimicrobials and disinfectants (803). Additionally evidence has been presented that illustrates that *qacA* has evolved from *qacB* (803). Similarly, it has been postulated that *qacC* has evolved from *qacD* (612).

The *qac* genes are widely distributed in clinical *S. aureus* isolates (59, 63, 772), and have been reported in many food-associated staphylococcal species (420-422). Plasmid-encoded *qacA* and *qacB* genes have also been described in *S. epidermidis* (592), and in antiseptic-resistant coagulase-negative staphylococci (592). Other *qac* determinants conferring biocide resistance have also been identified in *S. aureus*. These include *qacG*, coding for the QacG exporter of the SMR superfamily (421), *qacH* also encoding a protein of the SMR superfamily (423), and *qacEΔ1* (529). The *qacE* gene was first detected in a *Klebsiella aerogenes* plasmid (803) and *qacEΔ1* is a defective version of *qacE* (563).

Although some plasmid-mediated heavy metal resistance is significant in clinical isolates (536), and is transferable by conjugation or transduction, this is not the case for biocides commonly used as disinfectants. Moreover, the used of certain biocides in clinical settings might contribute to the reduction of spread of antimicrobial resistance by decreasing the success of conjugative and transductive transfer (7, 165, 342, 808). Because of the above, and the level and degree of the changes in susceptibility associated with plasmid-mediated resistance, the role of plasmids in resistance to biocides is less significant than it is with antibiotics, and they have little impact on biocide effectiveness (342, 910).

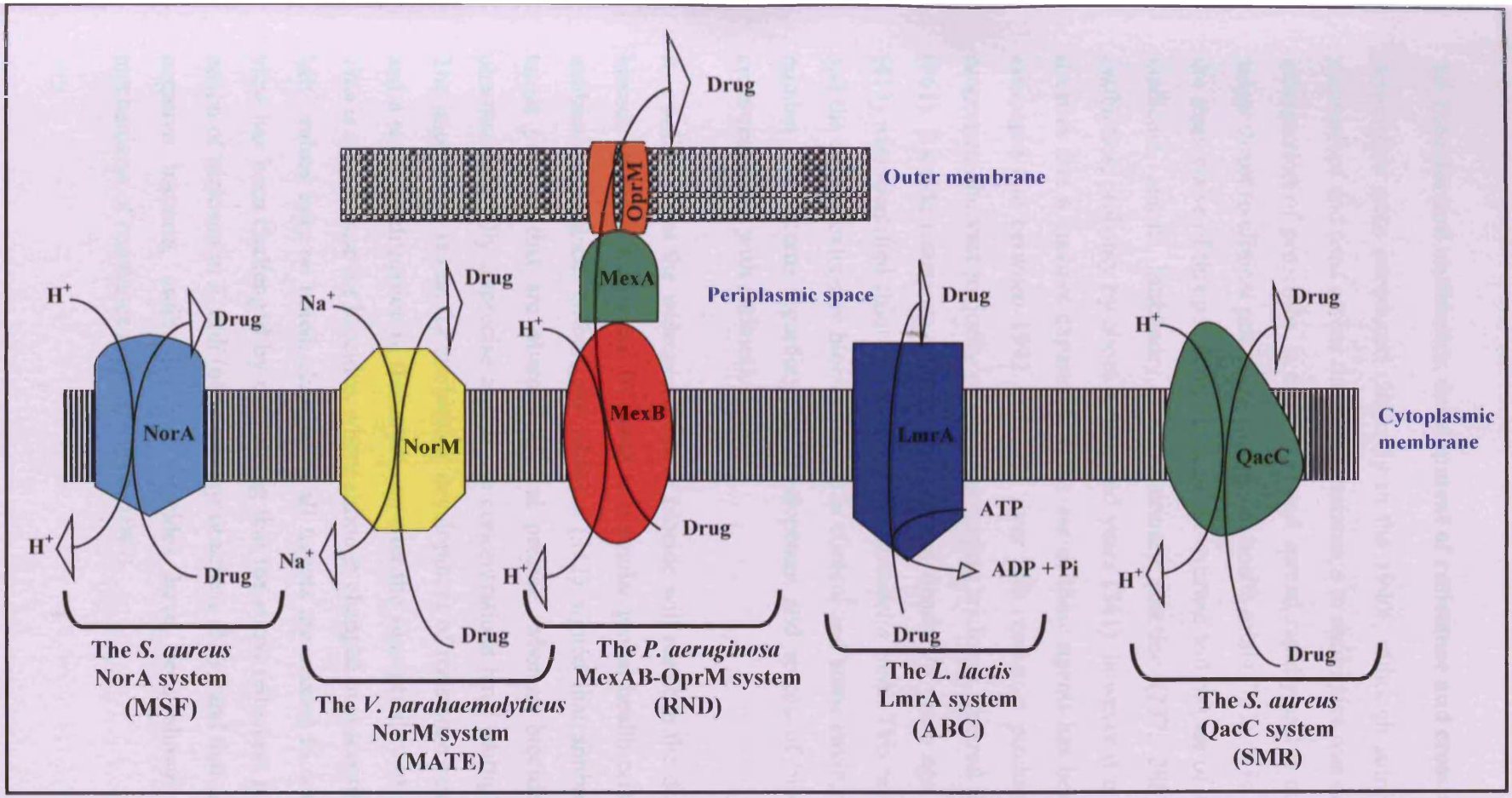


Figure 1.3. Schematic illustration of the main efflux pumps in bacteria. Adapted and modified from Schweizer (959) and Piddock (824).

Shown are: the NorA from *Staphylococcus aureus*, a member of the major facilitator superfamily (MFS), the NorM from *Vibrio parahaemolyticus* a member of the multidrug and toxic compound extrusion family (MATE), the MexAB-OprM system of *Pseudomonas aeruginosa*, a members of the resistance-nodulation-division family (RND), the LmrA pump of *Lactobacillus lactis*, members of the ATP-binding cassette family (ABC) and the *S. aureus* QacC system, a member of the small multidrug resistance family (SMR), now described as part of a larger drug/metabolite transporter superfamily (DMT) (483).

1.6 Biocides and antibiotics, development of resistance and cross resistance

Antibiotics were introduced clinically in the 1940s, although sulphonamides had been synthesised and used earlier than that. Resistance to antibiotics was noted shortly after the introduction of penicillin. It developed and spread rapidly and is currently viewed as a major threat to clinical practice and a global health crisis (753). It is widely accepted that the main cause of this problem has been the overuse and misuse of antibiotics in clinical medicine, animal husbandry, and veterinary practice (237, 288). Biocides predate antibiotics, probably by about a thousand years (341), however it is only in the last few decades that a massive expansion in the use of these agents has been taking place. It is estimated that between 1992 and 1999 over 700 consumer products with antibacterial properties, the vast majority of them containing triclosan, entered the consumer market (961). Biocide resistance was first recognized nearly 70 years ago by Heathman *et al.* (413) who identified chlorine resistance in *Salmonella typhi*. This resistance is on the rise and the current extensive biocides usage in clinical and home environments have raised a number of concerns regarding the development and spread of biocide resistance, and cross-resistance with antibiotics.

It is unlikely that the widespread use of biocide will result in the development of multi-biocide resistant strains and will lead to a similar global health crisis as that caused by antibiotic resistance. Gilbert and McBain (342) argued that antibiotics are considered target poisons that are pharmacological precise, whereas biocides are broad-acting, pharmacologically imprecise and at use concentrations have multiple target sites (457). The implication is that for antibiotics, development of resistance can be by a single step, and a small modification in the target can alter the susceptibility of the whole organism. This is not the case for biocides, where although changed in susceptibility as indicated by MIC values may be noted, changes in all targets are needed to confer resistance. This view has been challenged by the finding that the enoyl reductase is a target site for the action of triclosan in *E. coli* (685) and mycobacteria (683), and that with respect to Gram-negative bacteria, antibiotics and biocides have been shown to share common mechanisms of resistance (166, 834, 895, 1049).

Another concern about biocide and antibiotic resistance in bacteria is that antibiotic-resistant strains are also more resistant to biocide than sensitive ones, and vice versa, and that subtle differences in the biocide and antiseptic susceptibility facilitate selection and maintenance of these resistant strains in the environment. A number of studies have shown that antibiotic-resistant bacteria are not generally more resistant to in-use biocide concentrations than the corresponding sensitive bacteria (13, 16, 33, 805, 806, 897, 903, 907, 923, 927, 1030). However, others reported that there was a relationship between the degree of antibiotic resistance and resistance to biocide in bacteria (8, 9, 33, 121, 189, 713, 869, 1030, 1031). Moreover, bacteria showing reduced susceptibility to biocides may or may not be more resistant to antibiotics (1031, 1049, 1050).

Concerns over the extensive use of biocides in the environment have been growing, and it was suggested that biocide residues could lead to development of resistant strains. The evidence for this is also not conclusive. For instance, several reports have described isolates, especially among Gram-negative species, from various food processing environments that possess a reduced susceptibility to chlorine and quaternary biocides that relates to practical usage (435, 1149). However, opposing results have also been reported (313, 588) which suggested that there was no link between the use of biocides in environment and clinical settings and resistance.

It is not clear whether biocides select for antibiotic resistance in bacteria and if that could lead to emergence of cross-resistance. According to Gilbert and McBain (341), bacterial exposure to sub-effective concentrations of antibacterial agents means that the number of susceptible targets in the bacterium are reduced. At some point in the biocide concentration gradient there will be selection pressure on a single target in the organism. If that target happens to be shared with another party agent like an antibiotic, then there is a possibility that coincidental resistance to the antibiotic could arise. This is because changes in that one target might be enough to confer resistance to the antibiotic. There is scientific support for this idea. For instance, cationic biocides such as the polyhexamethylene biguanides and bisbiguanides, get into bacteria by a process called 'self-promoted uptake', in which the biocides displace cations in order to damage the cell and to get themselves in (341). Aminoglycoside antibiotics are known to use the same

mechanism of cell entry (397, 1039). Therefore, if a cell adapts itself and becomes less susceptible to a biguanide, it might in turn become less susceptible to an aminoglycoside.

Triclosan is also a good example of how shared targets between antibacterial agents could lead to development of cross-resistance. The mycobacterial InhA was shown to be a common target for triclosan and isoniazid in *M. smegmatis* (683) as well as a target for hexachlorophene (410) and for an experimental group of new antibiotics, the diazoborines (36). Moreover, cross-resistance between triclosan and other antimicrobial agents including trimethoprim, chloramphenicol, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride and chlorhexidine, has been reported (93, 94). It is still however not clear, at least from a clinical aspect, if the introduction of triclosan as an antimicrobial agent, played a role in the development of isoniazid resistant mycobacterial strains, or if the concerns over cross-resistance with this biocide are unfounded. For instance, *M. tuberculosis* is known to be intrinsically triclosan resistant but usually susceptible to isoniazid. Moreover, two InhA mutations (I47T and I21V) found in isoniazid-resistant clinical isolates of *M. tuberculosis* remained sensitive to triclosan (790).

The wide spread use of cationic biocides such as chlorhexidine and QACs can result in the selection of bacteria that are not only intrinsically insusceptible to these biocides but are also highly resistant to several chemically unrelated antibiotics. Strains of *P. aeruginosa, P. mirabilis, P. stutzeri, P. stuartii* and *S. marcescens* resistant to chlorhexidine and/or QACs were also resistant to several antibiotics (1018-1021, 1049, 1050). It was also suggested that the QAC, benzalkonium chloride, induced the expression of *qacA* and *qacB,* which confer low-level resistance to cationic biocides, and that their chronological emergence in clinical isolates of *S. aureus* mirrored the introduction and usage of cationic biocides in hospitals, notably acriflavine, diamidines, QACs (benzalkonium chloride, cetrimide) and chlorhexidine (799). Benzalkonium chloride-insusceptible staphylococci have been shown to be more resistant than sensitive ones to some antibiotics (979), and *S. aureus* MRSA strains trained to QAC resistance were shown to have increased resistance to several β-lactam antibiotics (5). Moreover a

link has been claimed between QACs and dyes resistance and insusceptibility to ampicillin and penicillin in clinical isolates and food-related staphylococci (979, 980).

Development of cross-resistance can also be achieved by efflux mechanisms which do not require change in the target site. Cells exposed to sub-lethal levels of antibacterial agents could select for mutants that are permanently switched on for some of their efflux pumps. These efflux mutants can pump antibiotics and many other agents out of the cell. Hence, when pumps are on, this can be sufficient to confer resistance to many distinct chemical agencies, making the efflux mutants multiply resistant. It was shown that in *E. coli* some efflux pumps are induced by exposure to sub-lethal levels of antibiotics, such as tetracycline, as well as QACs, triclosan, pine oil or salicylic (719). Upregulation of the AcrAB-TolC efflux system of *E. coli,* which pumps out pine oils, organic solvents, triclosan, QACs, chloroxanol, and chlorhexidine (684), lead to resistance to antibiotics, fluoroquinolones, ampicillin, tetracycline, nalidixic acid and chloramphenicol (684, 719). Sulavik *et al.* (1029) found that deletions in *tolC* or *acrAB* resulted in increased susceptibilities to the majority of the 20 different classes of antimicrobial compounds they studied which included antibiotics, antiseptics, detergents, and dyes. Multidrug efflux pump selecting for biocide and antibiotic resistance was reported in other organisms. Chuanchuen *et al.* (166, 168) reported that triclosan is a substrate for multidrug efflux pumps in *P. aeruginosa*, and that exposure to triclosan in a triclosan-sensitive *P. aeruginosa* mutant switched on an efflux pump that rendered the cell highly resistant to ciprofloxacin.

While the possible link between biocide use and antibiotic resistance has been demonstrated in laboratory *in vitro* studies, there is currently little direct evidence that this is significant in development of antibiotic resistance in clinical practice or the environment (670). Nevertheless, Gilbert and McBain (341) suggested that limiting our use of antibacterial agents to applications where there is proven gain and need for hygiene and using agents that lose their effectiveness rapidly once they are diluted from the point of action, will prevent build up of these agents in the environment. This will

help prevent development of resistance and cross-resistance with other antimicrobial agents.

Table 1.1. Biocide families, mode of action and mechanisms of resistance. Adapted from Poole (833), Maillard (646), Lambert (577), Moore (722) and Russell (899, 912).

Agent	Introduction/ application	Examples of uses	Known targets and mode of action	Resistance mechanisms
Biguanides			*Cell outer layers, cytoplasmic membrane, cytoplasmic constituents*	*Inactivation of biocide*
Chlorhexidine	1954	- Antiseptic - Disinfectant - Pharmaceutical preservative	- Binding to phosphate head groups and fatty acid chains in phospholipids - Membrane damage - Leakage of cellular constituents - Inhibition of enzyme activity - General cytoplasmic coagulation	- Chlorhexidine degrading activity (778) *Impermiability* - Changes in surface hydrophobicity (1048, 1049) - Changes in Omps (323) *Efflux* - CepA (281)
QACs BAC CPC Cetrimide	1933	- Skin disinfectants - Antiseptics - Pharmaceutical preservatives	*Cell outer layers, cytoplasmic membrane, cytoplasmic constituents* - Binding to phosphate head groups and fatty acid chains in phospholipids - Effect on membrane potential and electron transport chains - Membrane damage - Leakage of cellular constituents - Inhibition of enzyme activity - General cytoplasmic coagulation	*Impermiability* - Changes in Omps and fatty acids content (383, 384) - Surface change and hydrophobicity (623, 1049) - Changes in cytoplasmic membrane fatty acid content (623) *Efflux* - Qac(A-H) systems (421, 423, 592, 613, 676, 798, 802) - NorA system (507, 772) - SugE system (169) - EvgA system (771) - EmrE system (770, 1181) - AcrAB-TolC system (770)

Table 1.1. Biocide families, mode of action and mechanisms of resistance (continued). Adapted from Poole (833), Maillard (646), Lambert (577), Moore (722) and Russell (899, 912).

Agent	Introduction/ application	Examples of uses	Known targets and mode of action	Resistance mechanisms
Aldehydes			*Cell outer layer (well call) and cytoplasmic constituents*	*Inactivation of biocide*
Formaldehyde	1894	- Viricidal agents - Endoscope disinfection	- Cross linking - Interaction with amino groups in proteins and nucleic acids - Inhibition of enzymes and nucleic acid function	- Formaldehyde dehydrogenase activity (521, 569, 1157) *Impermiability* - Cell wall polysaccharide changes (656) - Outer membrane changes (28)
Glutaraldehyde	1960s			
OPA	1994			
Bis-phenols	1927		*Cell outer layers (outer membrane), cytoplasmic membrane, cytoplasmic constituents*	*Target site alteration*
Triclosan	Early 1970s	- Body washes - Dental hygiene	- Affect membrane potential and electron transport chains - Affect cytoplasmic and membrane proteins - Membrane damage - Cytoplasmic coagulation - Enzyme inhibition	- Mutation in fatty acid biosynthesis *fabI* gene and its homologue *inhA* (410, 683, 685, 790) *Degradation of biocide* - Production of triclosan degrading enzyme (689) *Efflux* - MexAB-OprM system (960) - MexCd-OprJ system (166) - MexEF-OprN system (166) - MexJK system (168, 961) - AcrAB-TolC system (770) - TriABC system (1043)

BAC; benzalkonium chloride, CPC; cetylpyridium chloride, OPA; *ortho*-phtalaldehyde, Omp; outer membrane protein

CHAPTER II

MOLECULAR BASIS OF BIOCIDE RESISTANCE AND SUSCEPTIBILITY IN MYCOBACTERIA

ABSTRACT

Some rapidly growing mycobacterial species such as *Mycobacterium chelonae* and *M. abscessus* are problematic opportunistic pathogens that frequently cause nosocomial infections due to their ability to contaminate solutions or devices. Mycobacterial resistance and susceptibility to antibiotics is well known, however, very little is known about the mechanisms of mycobacterial resistance and susceptibility to biocides. Agents such as triclosan (TRI), chlorhexidine diacetate (CHX), quaternary ammonium compounds and aldehydes are widely used in the home and clinical settings as a mean to disinfect instruments and clean surfaces. This study aimed to identify the molecular basis of biocide resistance and susceptibility in *M. smegmatis* mc^2155 (a model rapidly growing strain for which the complete genome sequence is available), and in *M. chelonae* and *M. abscessus* type strains (NCTC 946 and ATCC 19977 respectively). All mycobacteria grew well on Middlebrook 7H11 agar and in Middlebrook 7H9 broth, both supplemented with 0.5% glycerol and 10% OADC. *M. smegmatis* mc^2155 is late pigmented, and grew after 24 h incubation at 37°C. TSB, Lemco, and Sauton liquid media as well as TSA all supported the growth or this strain. *M. chelonae and M. abscessus* type strains were grown at different temperatures (30°C and 37°C respectively), and grew after 4 days incubation. All mycobacterial strains investigated were shown to have a typical bacterial growth curve. The growth inhibitory and killing effects of four biocides; TRI, cetylpyridinium chloride (CPC), CHX and alkaline *ortho*-phthalaldehyde (OPA) on the mycobacterial strains were investigated. From the agar Minimal Inhibitory Concentration (MICs) of the biocides it was shown that for *M. smegmatis* mc^2155, CHX, TRI, and CPC were most effective at inhibiting bacterial growth with MICs of 0.75 μg/ml, 1 μg/ml and 5 μg/ml respectively. OPA demonstrated much less inhibition, possessing MIC value of 2000 μg/ml. Agar MICs for *M. chelonae* type strain showed that CHX with MIC of 3.75 μg/ml was most affective, followed by CPC and TRI with MICs of 22.5 μg/ml and 30 μg/ml respectively. OPA was again the least effective agent at inhibiting growth of the *M. chelonae* strain and had MIC value of 2250 μg/ml. A similar phenotype was observed with *M. abscessus* type strain. CHX was the most effective at inhibiting growth of the strain and had an MIC of 8.75 μg/ml. It was followed by CPC and TRI both of which had MIC value of 25 μg/ml, and lastly OPA (MIC = 2250 μg/ml). Antibiotic susceptibility profiles for the mycobacterial strains were also determined and showed that *M. smegmatis* mc^2155 was resistant to ceftazidime, azithromycin, and piperacillin, but was susceptible to amikacin, tobramycin, ciprofloxacin, imipenem, meropenem, and trimethoprim-sulfamethoxazole. *M. chelonae* NCTC 946 was resistant to amikacin, ceftazidime, meropenem, azithromycin, and trimethoprim-sulfamethoxazole, but was susceptible to tobramycin and ciprofloxacin. *M. abscessus* ATCC 19977 was resistant to tobramycin, ceftazidime, azithromycin, meropenem, ciprofloxacin, and trimethoprim-sulfamethoxazole, and was only susceptible to amikacin. To determine the genes involved in susceptibility to the four biocides, 3000 *M. smegmatis* mc^2155 mutants (generated using a *mariner*-based transposon mutagenesis vector pM272B) were screened on agar containing a concentration of biocide just below its respective MIC. The screen failed to isolate any *M. smegmatis* mc^2155 mutants with altered biocide susceptibility. A previously characterised *M. smegmatis recA* mutant (HS42) and 6 transposon mutants form the *M. smegmatis* mc^2155 library with possible cell surface alteration, were selected for more detailed screening. The 6 mutants (10-A1, 2-B8, 15-E3, 3-A10, 2-A5, and 14-H1) showed different colony morphologies compared to the wild type strain, and had a drier and waxier appearance compared to the parental strain, suggesting possible alteration in cell surface composition. However, the 6 mutants as well as the *M. smegmatis recA* mutant did not show alteration in susceptibility to CPC or CHX compared with the wild type.

2.1 INTRODUCTION

2.1.1 Mycobacteria

The generic name *Mycobacterium* was first established by Lehmann and Neumann in 1896 (594) to describe the tubercle and leprosy bacilli pathogenic to man. The genus *Mycobacterium* is currently the only genus in the family Mycobacteriaceae, although recent studies based on 16S rRNA analyses have suggested that the family Mycobacteriaceae should include the three genera: *Mycobacterium*, *Nocardia*, and *Rhodococcus*. Mycobacteriaceae family constitute along with the families: Corynebacteriaceae (595), Dietziaceae (1002), Gordoniaceae (1002), Nocardiaceae (144), Tsukamurellaceae (1002), and Williamsiaceae, the suborder Corynebacterineae (1002) placed under the order Actinomycetales (123).

Mycobacteriacese are Gram-positive, non-sporing, acid fast, straight or slightly curved bacilli (44). They are non-motile and range in size between 0.2-0.6 μm in diameter to 1.0-10 μm in length. Both branching and mycelium-like growth may occur. They are generally aerobes and produce catalase. Many species form white or creamy coloured colonies but some form bright yellow or orange colonies based on carotenoid pigments, in some cases only as a response to light. The definition of the family Mycobacteriaceae and its unique genus is based on three main criteria: (1) acid-alcohol fastness, defined as the resistance to decolourization by acidified alcohol after staining with fuschin dye; (2) the presence and composition of the mycolic acids (section 2.1.7.1.2.3); (3) Guanin + Cytosine (G+C) content, which (with the exception of *M. leprae* which has a G+C content of 56%) is high in all mycobacteria ranging between 61% and 71% (976).

To date, over 100 mycobacterial species are included in the Approved Lists of Bacterial Names (http://www.bacterio.cict.fr) and many more may be isolated, identified and classified in the future especially with the introduction of powerful molecular classification techniques such as PCR restriction fragment length polymorphism (RFLP) analysis (1051).

2.1.2 Rapidly growing mycobacteria (RGM)

2.1.2.1 Taxonomy, description and characteristics

Except from the *M. tuberculosis* complex and *M. leprae*, other mycobacteria are referred to collectively as nontuberculous mycobacteria (NTM). Other names used for this group of organism include "environmental mycobacteria", "atypical mycobacteria", and "mycobacteria other than tuberculosis", although the term NTM is preferred (137, 1101). The traditional Runyon Classification System (based on major phenotypic features) (893), recognizes four groups of NTM. Groups I, II, and III, are considered slow growers (slow growing mycobacteria, SGM), with growth culture time similar to that of *M. tuberculosis*, whereas group IV consists of organisms which will grow well in routine bacteriologic media in less than 7 days, and they are collectively termed "rapidly growing mycobacteria" (RGM) (Figure 2.1).

2.1.2.2 Habitats

RGM are considered saprophytes, normally found in soil, water and dust in natural ecosystems (278). However human activities and human interaction with the environment have had a big impact on the RGM bacterial populations and helped in some cases to extend their natural habitats. For instance, constructed environments provided new surfaces for adherence and growth, particularly for bacterial adapted to aquatic ecosystems. RGM grow in water including distilled water, at water surfaces (278, 1106), and have been detected in municipal water supplies and water systems (109, 142, 197, 278). Tolerance to high temperatures (958, 1106) and chemical disinfectants such as chlorine and glutaraldehyde (197, 1106) have contributed to the ability of RGM to invade extremely hostile environments (1129, 1155, 1156).

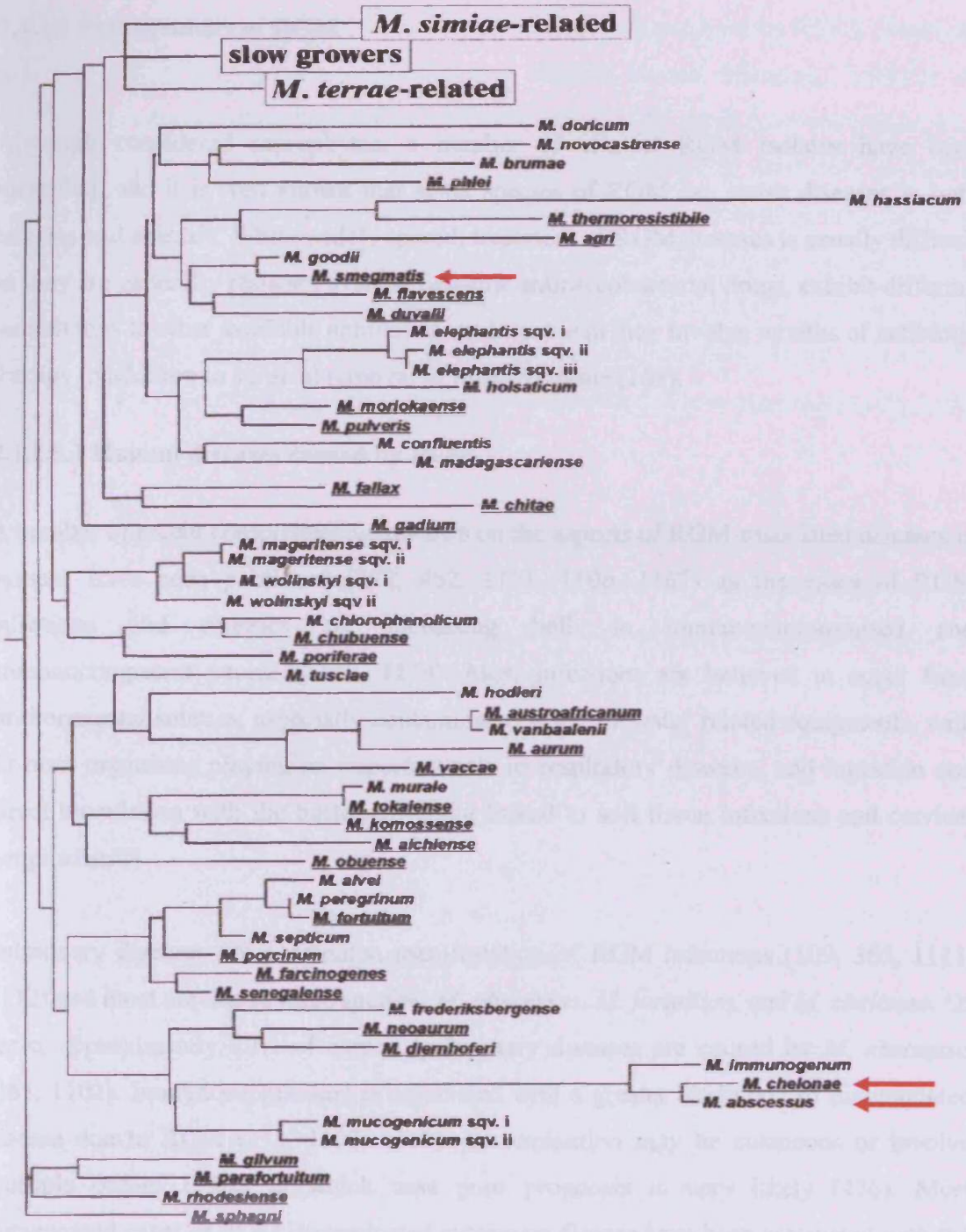


Figure 2.1. Phylogenetic tree of rapid growing mycobacteria

The tree includes new mycobacteria along with previously defined species (underlined). It is based on the 16S rDNA nucleotide sequence. Arrows point to the mycobacterial species investigated in the current study. Adapted from Tortoli (1064).

2.1.2.3 Pathogenicity of RGM

Although considered saprophytes, a number of clinical RGM isolates have been identified, and it is well known that some species of RGM can cause diseases in both humans and animals. While widely spread, treatment of RGM diseases is usually difficult as they are generally resistant to most first-line antimycobacterial drugs, exhibit differing sensitivities to other available antibiotics, and treatment may involve months of antibiotic therapy in addition to surgical removal of infected tissue (108).

2.1.2.3.1 Human diseases caused by RGM

A number of recent comprehensive reviews on the aspects of RGM-associated diseases in humans have been published (278, 452, 1101, 1106, 1167) as the cases of RGM infections and diseases are increasing both in immunocompromised and immunocompetent people (1167, 1179). Most infections are believed to occur from environmental sources, especially contaminated water or water related equipments, with air-born organisms playing an important role in respiratory diseases, and ingestion and direct inoculation with the bacterium being linked to soft tissue infections and cervical lymphadenitis.

Pulmonary diseases are a common manifestation of RGM infections (109, 365, 1111, 1112) and most are due to three species: *M. abscessus*, *M. fortuitum*, and *M. chelonae*. Of these, approximately 80% of chronic pulmonary diseases are caused by *M. abscessus* (365, 1102). Immunosuppression is associated with a greater likelihood of disseminated disease due to RGM (278, 1107, 1167). Dissemination may be cutaneous or involve multiple organs (278), in which case poor prognosis is very likely (476). Most documented cases of RGM disseminated cutaneous disease have been associated with the *M. abscessus-chelonae* group (section 2.1.5) and *M. fortuitum* (85, 160, 451, 1115, 1154). However, although disseminated diseases due to RGM are usually associated with immunorepression, there have been numerous reports of diseases in otherwise healthy patients (160, 476, 751).

Cutaneous, soft tissue, and bone diseases are also commonly caused by RGM. Infections generally result from posttraumatic and post surgical wound infections, insertion of medical devices, and other medical procedures (1105, 1115). These have been associated with a number of sporadic nosocomial or health care-associated infections including disease that involved renal dialysis, punch biopsy surgery, augmentation mammaplasty, other forms of plastic surgery including face-lifts and liposuction, sternal wound infections following cardiac surgery, and postinjection abscesses (85, 127, 696, 1096, 1110, 1113). Catheter-related infections can also be caused by RGM and they are the most common form of health care associated disease due to these organisms (109, 1107, 1111, 1112, 1114, 1151).

Bone and joint infections are a frequent complication due to RGM infections where osteomyelitis can develop due to open bone fractures, puncture wounds, and hematogenous spread from another source. The most common pathogen in this setting is *M. fortuitum,* although other RGM have been involved (109, 149, 278, 695, 1101, 1107, 1114, 1167).

2.1.2.3.2 Animal diseases caused by RGM

In animals, diseases caused by RGM are similar in type to those in humans (section 2.1.2.3.1). These are mainly opportunistic localized infections, such as through bite wounds (377), due to the low pathogenicity of these organism in clinically normal animals, although disseminated disease in animals have also been reported (302, 377). For instance, disseminated granulomatous lesions in fish, tuberculosis-like lesions in the lungs of turtles, and abscesses and ulcerative lesions in manatees (847, 1056), have all been reported and linked to *M. chelonae.* Other infections such as granulomatous lesions which occur in mammals skin, lungs, lymph nodes, and joints infections are also common (847). Granulomatous mastitis in cattle and ulcerative skin lesions in cats due *M. smegmatis* infection, and the less common ulcerative skin lesions in cats from *M. phlei* have also been reported (847). Some RGM appear to be exclusively animal pathogens,

including both *M. porcinum,* which causes lymphadenitis in pigs, and an agent of bovine farcy (976).

2.1.3 *M. smegmatis* and the *M. smegmatis* group

2.1.3.1 Description and characteristics

Mycobacterium smegmatis was first described in 1885 by Lustgarten as a cause of syphilitic penile ulcers (629). Although subsequently shown to have no relationship to syphilis, the organism was named for the genital secretions (smegma) from where it was isolated, and first designed "*Bacillus smegmatis*" (1072), then "*Mycobacterium smegmatis*" (596). The first modern day survey of isolates was in 1953 (355) and described 56 strains, all from culture collections and none from human sources. Gordon and Smith (355), studied 124 isolates and reported that out of the 124 isolates studied 56 strains received as 12 different species, all belonged to one species, *M. smegmatis*. Gorden and Smith (355), gave a detailed description of the *M. smegmatis* isolates studied. The colonies of *M. smegmatis* were either dense with smooth edges, dense fringed with filaments or completely filamentous. The actual organisms were acid fast, usually slender of varying lengths, curved and beaded and grew at temperatures from 28^0C to 45^0C but not 60^0C. Biochemically the organisms formed acid from glucose, rhamnose, xylose, arabinose, sorbitol, inositol, mannose, and galactose, hydrolyzed starch but not casein, most reduced nitrates to nitrite, and grew in 5% NaCl but not 7%.

In 1988, Wallace *et al.* (1111) reported the first clinical isolates of *M. smegmatis*, when they characterised 22 isolates from infections and proposed the existence of three groups in the species *M. smegmatis*. Subsequent genetic and molecular studies confirmed the heterogeneity in *M. smegmatis* isolates (1008, 1051, 1111), and Brown *et al.* (109), named the three *M. smegmatis* groups as *Mycobacterium goodie* sp. nov., *Mycobacterium wolinskyi* sp. nov., and *Mycobacterium smegmatis* sensu stricto.

2.1.3.2 *M. smegmatis* sensu stricto

2.1.3.2.1 Colonial and biochemical characteristics

M. smegmatis show similar characteristics to the *M. smegmatis* group it belongs to (Table 2.1). They are acid fast rods which produce irregular colonies with lobate margins which are dry, flat, and appear waxy due to the high concentration of lipids in the cell wall. Over 90% of the *M. smegmatis* colonies produce a late yellow-orange pigmentation on Middlebrook 7H10 agar (109). Because the pigmentation is so late and usually occurs only on select media, it is often missed.

Table 2.1. Biochemical characteristics of *M. smegmatis*. Adapted from Brown *et al.* (109, 1103) and Wallace *et al.* (109, 1103).

Characteristic	*M. smegmatis* ATCC 19420 type strain
Smooth colony	+
Growth at 50°C in 3 days	-
Growth at 45°C in 3 days	+
Pigmentation*	+
3-day arylsulfatase	-
p-aminosalicylate degradation	-
Nitrate reduction	+
Iron uptake	+
Growth in presence of 5% NaCl	+
Thermostable catalase (68°C)	-
L-arabinose**	+
Citrate**	+
D-galactose**	+
D-trehalose**	+
D-xylose**	+
D-mannitol	+
D-rhamnose	+

* Pigmentation after more than 10 days incubation in Middlebrook 7H10 agar, **Assimilation.

2.1.3.2.2 Drug susceptibility

M. smegmatis as other members of the *M. smegmatis* group, is susceptible to sulfonamides, doxycycline, imipenem, amikacin, and 5 μg/ml ethambutol. The organism is however resistant to 25 μg/ml rifampin, and 10 μg/ml isoniazid (Table 2.2).

Table 2.2. MICs of *M. smegmatis* to a range of antibiotics as determined by broth microdilution. Adapted from Brown *et al.* (109, 1111) and Wallace *et al.* (109, 1111).

Antibiotic	MIC* range	MIC^*_{50}	MIC^*_{90}
Aminoglycosides			
Amikacin	≤0.25-0.5	≤0.25	0.5
Gentamycin	≤0.25-2	0.5	2
Tobramycin	≤0.25-1	≤0.25	1
Kanamycin	≤0.25-1	0.5	1
Streptomycin	≤0.5-1	≤0.5	1
β-lactams			
Cefoxitin	8-64	16	64
Cefmetazole	4-32	8	32
Imipenem	4	4	4
Anti-tuberculosis			
Isoniazid	>16	>16	>16
Rifampicin	>16	>16	>16
Ethambutol	≤0.5– 1	≤0.5	1
Ansamycin	4	4	4
Others			
Ciprofloxacin	0.5-1	0.5	1
Doxycycline	≤0.25	≤0.25	≤0.25
Erythromycin	>16	>16	>16
Sulfamethoxazole	≤1	≤1	≤1

* Expressed in μg/ml, MIC_{50}; MIC which inhibits 50% of strains tested, MIC_{90}; MIC which inhibits 90% of strains tested.

2.1.3.2.3 Pathogenicity of *M. smegmatis*

Although for its first 85 years it was regarded as a saprophytic organism of no clinical significance, *M. smegmatis* is now known to be involved in a number of both human and animal diseases and infections. In humans the first well described case was reported in 1986 (1098) and involved the lung and pleura in a patient with underlying lipoid pneumonia. Since then, *M. smegmatis* has been incriminated in a number of human diseases including community-acquired and post-traumatic wound infections (109, 831, 874, 875, 1111, 1156), and rarely, respiratory diseases, usually associated with exogenous lipoid pneumonia (109, 1111). The organism has been recovered from a number of health care associated infections, including sternal wound sites following cardiac surgery, breast abscess following augmentation mammaplasty (109, 1111), and bacteremia from intravenous catheter placement. A case of the latter was reported in 1998 by Skiest *et al.* (987), where *M. smegmatis* was reported to be the cause of a central venous catheter-related bacteremia in a cancer patient. Skiest *et al.* (987) suggested that *M. smegmatis* should be added to the list of RGM that are capable of causing catheter infections.

In animals *M. smegmatis* is currently recognized as an aetiologic agent of bovine mastitis (874, 956, 1058). *M. smegmatis*-induced granulomatous mastitis was seen in a diary herd after intramammary treatment (1058). Recently the organism has been implicated in systemic granulomatous lesions in an immunocompromised dog (377), and in infections in frog (43, 277, 851, 974). In 1999, Talaat *et al.* (1046) reported for the first time evidence that *M. smegmatis* is also pathogenic to fish, when the pathogenicity of *M. smegmatis* was compared with that of the known fish pathogen *M. fortuitum*, in a goldfish model of infection.

2.1.4 Genetics of *M. smegmatis*

2.1.4.1 *M. smegmatis* as a model organism

The application of molecular genetics in the study of mycobacteria has allowed a dramatic increase in the number of studies investigating mycobacterial genes and their protein products. Therefore, led to a better understanding of mycobacterial life, especially in relation to human pathogens such as *M. tuberculosis* and *M. leprae*. The publication of the full genome sequences of these two major human pathogens (182, 183), have dramatically improved our understanding of their metabolism, biochemistry, and pathogenicity. However, with all the advances in genetic and molecular techniques, genetic analysis of mycobacteria has lagged considerably behind that of other prokaryotes. This is due several reasons: First the slow growth rate of many of mycobacterial species, especially those with clinical significance, which precludes many approaches to selection or screens that are widely used with other bacteria; Second, the tendency of mycobacteria to grow in clumps due to their complex cell wall composition, prevents single-cell suspensions, hence the isolation of colonies derived from single cells; Third, lack of gene transfer systems, and convenient methods for mycobacterial genetic analysis, even though some have been developed recently; Finally, for many mycobacterial species, the inconvenience of working with an intractable organism under biosafety level III has also contributed to the slow advance of mycobacterial genetics.

A useful way for studying mycobacteria, especially pathogenic species, is to use model organisms such as *M. smegmatis*, a non-pathogenic, rapid growing mycobacteria, to study gene function and product in those pathogens. *M. smegmatis* has the advantage over the more common genetic hosts, such as *E. coli*, in that it is a homologous host to mycobacterial genes. For instance, genes of mycobacterial strains that are difficult to grow, such as *M. tuberculosis* or *M. leprae*, could be cloned into *M. smegmatis* host where they are likely to be expressed. This would also eliminate the use of exogenous promoters to express these genes. The inability of *M. smegmatis* to be efficiently

transformed with plasmid vectors has been improved by the isolation of the efficient plasmid transformation mutant mc^2155 by Snapper *et al.* (993).

The genome sequencing of *M. smegmatis* mc^2155 has been completed at the J. Craig Venter Institute (previously The Institute for Genomic Research, TIGR) (http://www.tigr.org). The genome is 6988209 bp, with a 67.4% C+G content, and its publication will undoubtedly increase the value of *M. smegmatis* as a model for the study of mycobacterial genetics. Generation, screening and analysis of *M. smegmatis* mutants offer a wealth of opportunities for the study of mycobacterial genes, proteins, and their functions. A large number of *M. smegmatis* mutants have been generated either spontaneously, by the use of mutagens or transposons, and a collection of these are summarized in Table 2.3.

Table 2.3. Summary of some of *M. smegmatis* mutants and their characteristics.

M. smegmatis mutant	Reference	Mutant characteristics
Mutant defective in biosynthesis of mycolic acids.	(571)	-Penicillin-sensitive -Smooth and filamentous colonies - Devoid of mycolic acids, but accumulated short chain fatty acids than those in the wild type.
Mutants resistant to fluoroquinolones, (*gyrA* defective)	(868)	
• Mutants A and C		-Low level resistance to fluoroquinolones
• Mutants C, D, and E		-High level resistance to fluoroquinolones
Mutants defective in siderophore production: mutants SM1 and SM3 (*ideR* defective).	(252)	-Incomplete derepression of siderophores mycobactin and exochelin production. -Increased sensitivity to H_2O_2. -Decreased levels of catalase/peroxidase KatG activity. -Decreased levels of manganese superoxide dismutase.
Isoniazid-resistant mutants (*katG* defective)	(79)	-Higher MIC for Isoniazid (100- 200 μg/ml) than parental strain mc^2155 (25 μg/ml). -Loss of catalase/peroxidase activity.
Mutant defective in inositol monophosphate phosphatase gene homolog: mutant LIMP7 (*impA* defective)	(791)	-Slower growth rate than the parent strain -Increased clumping in liquid culture -Formation of smaller colonies than the parent strain -Increased resistance to chloramphenicol and erythromycin. -Increased sensitivity to isoniazid and several β-lactams. -Altered cell envelope, by decrease in accumulation of lipophilic molecules and increased accumulation of hydrophilic molecules.
Mutants defective in DNA-replication gene (*dnaG* defective)	(546)	-Temperature-sensitive mutants -Fail to grow at 42^0C -Exhibit filamentous phenotype at nonpermissive temperature -Sensitive to mycobacteriophages TM4, D29, and L5
Mutants defective in NADH-dehydrogenase (*ndh* defective)	(700)	-Co-resistance to isoniazid and related drug ethionamide -Thermosensitive lethality -Auxotrophy

Table 2.3. Summary of some of *M. smegmatis* mutants and their characteristics (continued).

M. smegmatis mutant	Reference	Mutant characteristics
Mutant lacking penicillin binding protein1 PBP1: mutant MUT1 (*ponA* defective)	(78)	-More sensitive to β-lactam antibiotics than the wild type -Grows more slowly in liquid culture - More permeable to glycine
Mutants defective in 1-Dmyo-Inosityl-2amino-2-deoxy-α-D-glucopyranoside and mycothiol (MSH) biosynthesis.	(754)	
• Strains 5, 6, and 49		-More sensitive to H_2O_2
• Strain 49		-Devoid of MSH -Grows more slowly on solid media than the parent strain. -More sensitive to rifampicin. -Less sensitive to isoniazid.
Mutant defective in biosynthesis of mycolic acids 155NS1	(615)	-Hypersensitive to β-lactams, hydrophobic compounds such as novobiocin, rifampicin, erythromycin, chloramphenicol, and crystal violet. -Temperature sensitive -Devoid of mycolic acids, but accumulated novel fatty acids that are not detected in parent strain, such as meromycolates.
Mutant simultaneously resistant to D-cycloserine and to vancomycin (Cvr-1).	(818)	-Resistant to both D-cycloserine and to vancomycin -Resistant to other peptidoglycan biosynthesis affecting drugs such as fosfomycin.
Mutants defective in mycothiol production:		
• Strain 164 (*mshC* defective)	(859)	-Chemical mutant. -Low levels of MSH -Increased sensitivity to alkylating agents (chlorodinitrobenzene, monobromobimane, iodoacetamide, and diamide) -Increased sensitivity to H_2O_2 -Increased sensitivity to redox-cycling agents -Increases sensitivity to antibiotics (erythromycin, azithromycin, vancomycin, penicillinG, rifamycin and rifampicin). -Increased resistance to isoniazid.
• Mutants Tn1 and Tn2		-Transposon mutants similar characteristics to strain 164
Mutant hypersensitive to rifampicin: mutant RHS234 (*arr* defective)	(10)	-Hypersensitive to rifampicin -No altered sensitivity to other antibiotics
Mutant sensitive to diamide (defective in ORF Rv0274): mutant T7	(858)	-More sensitive to diamide than the wild type. -Sensitive to other thiol modifying agents such as iodoacetamide and chlorodinitrobenzene -Not sensitive to other oxidative stresses such as redox cycling radicals and organic peroxides.
Mutant resistant to triclosan and isoniazid (*inhA* defective)	(683)	- Increased resistance to triclosan - Increased resistance to isoniazid

2.1.5 *M. chelonae-abscessus* group

The *M. chelonae-abscessus* group contains three species: *M. chelonae, M. abscessus,* and *M. immunogenum* formerly *M. immunogen. M. immunogenum* is a recently described RGM isolated from contaminated metalworking fluids (720) and will not be discussed in this report.

M. chelonae was first isolated by Friedman in 1903 from the lung of a turtle identified at that time as *Chelonia corticata.* In 1972 Stanford (1004) first reported studies on clinical isolates of what was then known as *M. borstelense* (797), resulting in the official adoption of the name "*M. chelonei*" which was later changed to the more correct Latin, *M. chelonae. M. abscessus* was first isolated by Moore and Frerichs in 1953 from a human joint (knee abscess) and was given the name abscessus because of its ability to produce deep subcutaneous abscesses (721).

M. chelonae and *M. abscessus* were thought to be the same organism as they showed almost identical biochemical features. However, a cooperative numerical phenotypic study by the International Working Group on Mycobacterial Taxonomy (IWGMT) published in 1972, demonstrated that the two taxa were sufficiently different to be classified as subspecies and renamed them *M. chelonae* subspecies *chelonae,* and *M. chelonae* subspecies *abscessus* (561). This taxonomic status was held until 1992 when Kusunoki and Ezaki firmly established through DNA hybridization that these organisms had only 35% DNA homology and they are separate species (573). Biochemically only two tests, those for sodium chloride tolerance and utilization of citrate, are useful for identifying *M. chelonae* and *abscessus* organisms at the species level (982). Furthermore, conventional molecular taxonomy techniques relaying on sequence comparison of the 16S rDNA are of limited use to separate the two species because of the low variability in their rDNA gene (573). However, a number of other biochemical and molecular methods have successfully been used to distinguish the two species apart (132, 133, 431, 982, 1126, 1172, 1173).

2.1.5.1 *M. chelonae*

M. chelonae is a rapidly growing nontuberculous mycobacteria that is found in the environment and can cause human diseases. As mentioned above, the organism was first isolated in 1903 from sea turtle and normally exists as a free-living saprophyte in soil, water, and dust. *M. chelonae* grow to visible colony size within 7 days at temperatures ranging from 28^0C to 30^0C. It is one of the most antibiotic resistant species of pathogenic RGM. Although opportunistic, it can cause infections in patients with or without compromised immunity. The clinical outcome depends on the immune status of the host and the inoculation mechanism.

M. chelonae have been associated with a range of community-acquired infections including disseminated diseases of which disseminated cutaneous disease in chronically immunorepressed hosts are the most common (29, 1104, 1112). Localized infections are also common, and usually occur following trauma. In a study by Wallace *et al.* (1107) cases of localized cellulitis, subcutaneous abscess, or osteomyelitis developing after penetrative trauma, were reported to be exclusively due to *M. chelonae*. Other diseases have been linked to *M. chelonae* such as corneal ulcers, lymphadentitis, and a case of thyroid abscess in a 45-year old immunocompetent woman (787). Interestingly however, unlike *M. abscessus* and *M. fortuitum*, *M. chelonae* is only rarely a cause of pulmonary diseases. In a study by Griffith *et al.* (365) on a series of 154 patients with chronic lung disease due to RGM, only 1 of 146 isolates identified to species level was an *M. chelonae*.

Health care associated infections due to *M. chelonae* are not as common as those with *M. abscessus* or *M. fortuitum*. However, because *M. chelonae* is relatively chlorine-resistant and able to grow well and survive at relatively high concentrations in tap water and distilled water (278, 1096), it can cause a number of outbreaks following medical or surgical procedures such as injection with contaminated needles or solutions. Outbreaks of *M. chelonae* infections have been reported after injections with histamine (478), lidocaine (135, 1096), saline solution (364), vaccines (87), disinfectant solution (1138),

and adrenal cortex extract (322). Although only three of these reports were clearly ascribed to *M. chelonae* after the separation into two different species (87, 478, 1085). Other sources of infection included implantation of contaminated porcine heart valves (309, 487, 1106), treatment with acupuncture (18), and, recently, the use of liposuction (697). The other common type of health-care associated *M. chelonae* infection is that related to catheters (330). In another study by Wallace *et al.* (1107), it was found that 8 out of 100 clinical isolates of *M. chelonae* were associated with intravenous catheters, an additional 3 involved in chronic peritoneal dialysis catheters, and 1 involved a hemodialysis shunt.

2.1.5.2 *M. abscessus*

M. abscessus, originally isolated in 1953 from a human joint (721), is non-pigmented and form visible colonies within few days of growth, even on routine bacteriological substrates. It has an optimum growth temperature at 28^0C to 30^0C with colony morphology ranging from rough to smooth. *M. abscessus* is an environmental organism ubiquitously found in water, decaying vegetation, sewage water, as well as drinking water tanks and municipal tab water (108, 957), but is also known to cause nosocomial outbreaks and was isolated from hospital environments (650, 1106).

M. abscessus is considered the most pathogenic and chemotherapy-resistant RGM, and like *M. chelonae* is involved in a number of both community-acquired infections and health-care related diseases, with pulmonary diseases accounting for most clinical isolates of this species. A study by Griffith *et al.* (365) on 146 disease-associated pulmonary RGM isolates identified to species and collected over a 15-year period by a Texas reference laboratory, 82% were caused by *M. abscessus*. Patients with pulmonary disease due to *M. abscessus* usually have other underlying diseases which could contribute to lung damage, including bronchiectasis, cystic fibrosis (CF), gastroesophageal disorders, and prior granulomatous disease such as sarcoidosis or tuberculosis. After *M. avium* complex, *M. abscessus* is the second most common species of NTM recovered from respiratory specimens in patients with CF (203, 273, 782, 942).

M. abscessus causes a wide variety of extrapulmonary diseases as well as health care-associated ones. Wallace *et al.* (1115) studied a series of 59 nonrespiratory isolates belonging to the *M. chelonae-abscessus* group and found that *M. abscessus* cases were by far the most common. Among the 30 cases of nonpulmonary disease caused by *M. abscessus,* 43% were postsurgical or postinjection wound infections, 23% were localized community-acquired wound infections, 20% were disseminated cutaneous infections, and 13% were miscellaneous types of infections including keratitis and prosthetic valve endocarditis.

Along with *M. fortuitum, M. abscessus* is the most common RGM species causing nosocomial disease, especially sporadic and clustered outbreaks of surgical wound infections (1115). Disease outbreaks have also been described after augmentation mammaplasty (303), facial plastic surgery, liposuction (738), acupuncture (930), cardiac surgery, micrographic surgery (821), eye laser surgery (336), injections of alternative medicines, steroid injections, and miscellaneous types of surgery (308, 1192). Although unusual, disseminated diseases due to *M. abscessus* may develop and are serious especially in chronically immunorepressed patients where most of these cases occur. For instance Bolan *et al.* (85) reported 25 infections due to *M. abscessus* in a hemodialysis center where 9 of these patients had widely disseminated disease. Disseminated infections due to endocarditis with *M. abscessus* have been reported (867, 1115), and in one case the disseminated infection, which meningitis was part of, lead to the death of a patient with endocarditis caused by *M. abscessus* (608).

2.1.6 Antimicrobial susceptibility, therapy and treatment of diseases caused by RGM

Antimicrobial therapy for RGM, unlike that used for most SGM diseases, may vary depending on the nature of the disease, the host, and the infecting organism. This is further complicated by the fact that RGM are resistant to many drugs, the *in vitro* sensitivity of an antimicrobial agent may not completely reflect its *in vivo* efficacy (840), and development of resistance.

M. smegmatis group is uniformly susceptible to ethambutol, sulfonamides, imipenem, amikacin and doxycycline (109, 1111). They exhibit intermediate susceptibility to the older fluoroquinolones (ciprofloxacin and ofloxacin), variable susceptibility to cefoxitin and clarithromycin, and are resistant to isoniazid and rifampin (1111). The main antimicrobial agents used for treatment of diseases due to the *M. smegmatis* group are oxycycline and trimethoprim-sulfamethoxazole for oral use and the injectable agents amikacin and/or imipenem. Surgical intervention along with antimicrobial therapy might be necessary for treatment (755).

M. chelonae-abscessus group of organisms are characterised by their high resistance to antimicrobial agents, and treatment for diseases cause by these organisms often requires the use of multiple drug therapy and surgical intervention (1114). When foreign bodies such as wood splints, silicon implants, or percutaneous catheters are involved, removal of the foreign bodies appears to be essential to recovery (176, 205, 432, 625, 1101, 1102). *M. chelonae* and *M. abscessus* are resistant to antituberculous drugs, however they are susceptible to a number of other antimicrobial agents, which if used appropriately and in the right combinations can lead to effective therapy. In studies by Wallace *et al.* (1104, 1107, 1108), it was reported that 100% of *M. abscessus* isolates were susceptible to clarithromycin, approximately 90% were susceptible or intermediate to amikacin (32 μg/ml) and cefoxitin (64 μg/ml) and about 50% were susceptible or intermediate to imipenem (8 μg/ml) and linezolid (16 μg/ml). Isolates of *M. chelonae* were reported to be susceptible to amikacin (80%), tobramycin (100%), clarithromycin (100%), imipenem (60%), clofazimine and doxycycline (25%), and ciprofloxacin (20%).

Harnesing the differences in susceptibility of *M. chelonae* and *M. abscessus* to antimicrobial agents is crucial when it comes to choosing the appropriate drug treatment for infection. For instance, *M. chelonae* and *M. abscessus* have different susceptibility to tobramycin. In a study on 75 isolates of *M. chelonae* and *M. abscessus*, Yakrus *et al.* (1172) reported that all *M. chelonae* isolates were susceptible to tobramycin (MIC $\leq$4 μg/ml), whereas *M. abscessus* isolates were either resistant or intermediately resistant to

the drug (MIC of 8 to ≥16 μg/ml). Similarly, isolates of *M. chelonae* show resistance to cefoxitin (MICs ≥128 μg/ml) whereas *M. abscessus* isolates have MICs range from 16 to 64 μg/ml, with 32 μg/ml being the model MIC (1036).

2.1.7 Antimicrobial agents, activity, and resistance in mycobacteria

2.1.7.1 Mycobacterial cell envelope

Mycobacteria are resistant to a wide range of antibiotics and chemotherapeutic agents. This resistance among other mycobacterial characteristics such as their small size relative to other bacteria, their hydrophobicity and acid fast staining, are all thought to be related to the unusual structure and low permeability of their cell envelope. The latter is essential for growth and survival of mycobacterial cells in their host, therefore understanding the composition, functions and biosynthesis of the different cell envelope components should enable the development of effective drugs to combat mycobacterial infections. The mycobacterial cell envelope is composed of the cell wall overlying the cell cytoplasmic (plasma) membrane which surrounds the cytoplasm (Figure 2.2).

2.1.7.1.1 The cytoplasmic membrane

The mycobacterial plasma membrane is an asymmetric lipid (mainly phospholipids) bilayer which in its basic structure does not differ from that of other bacterial membranes with the addition of some distinctive components. The plasma membrane contains a number of phospholipids, the main ones are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol mannosides (PIMs) and the less common phosphatidylinositol (103). The lipids are assembled in the bilayer in association with proteins and other compounds including a number of polyterpene-based products thought to play a role in protection against photolytic damage (103), menaquinones which are involved in electron transport (102), and carotenoids. The latter vary in structure from species to species and if present in sufficient quantities can give the bacterial colonies a bright yellow colour. In photochromogenic mycobacteria,

such as *M. kansasii* carotenoid pigments are only synthesized on exposure to light, while in scotochromogens, such as *M. gadium* they are also produced in the dark.

2.1.7.1.2 The cell wall

The mycobacterial cell has a comparatively thick cell wall which consists of several components and has a covalently linked skeleton (711) as its core structure. This skeleton is the mycolyl-arabinogalactan-peptidoglycan complex (mAGP), 40% of which corresponds to lipids in the form of mycolic acids. The mAGP consists of an innermost layer of murein, or peptidoglycan covalently linked to the mycolyl-arabinogalactan (mAG) via phosphodiester bonds. The mAG is composed of a layer of arabinogalactan, a branched macromolecule composed of arabinose and galactose, which its side chains are linked to a layer of mycolic acids. In addition to the mAGP skeleton, mycobacterial cell wall contains various numbers of lipids and related compounds including lipoarabinomannan (LAM), glycolipids, and mycosides. Mycobacteria are not capsulated in the strict sense, however in some strains the surface mycoside layer is very thick and gives the colonies a smooth appearance.

2.1.7.1.2.1 Peptidoglycan

The mycobacterial peptidoglycan consists of alternating units of *N*-acetylglucosamine and *N*-glycolylmuramic acid (72). The tetrapeptide side chains, consisting of L-alaninyl-D-isoglutaminyl-meso diaminopimelyl-D-alanine (820) with the diaminopimelic acids being further amidated (1143), are attached to muramic acid which is cross-linked to arabinogalactan via a phosphodiester linker (589). This type of peptidoglycan (Alaγ) is one of the most common found in bacteria. However, mycobacterial peptidoglycan is distinct in that the muramic acid residues are *N*-glycolylated with glycolic acid, and the peptidoglycan cross-links include bonds between two residues of diaminopimelic acid as well as between diaminopimelic acid and D-alanine (335).

2.1.7.1.2.2 Arabinogalactan

The arabinogalactan polymer is comprised exclusively of D-galactofuranoses and D-arabinofuranoses residues which are extremely rare in nature. The polysaccharide is also unusual in the type of its component sugars as well as its overall structure (209). Unlike bacterial polysaccharides, mycobacterial arabinogalactan is composed not of repeating units but a few distinct, defined structural motifs. The arabinogalactan is attached to the peptidoglycan by its linear galactan via a diglycosylphosphoryl bridge. The mycolic acids are located in clusters of four on the terminal pentarabinofuranosyl units, but only two thirds of these are mycolylated (688).

2.1.7.1.2.3 Mycolic acids

Mycolic acids are high molecular weight (C_{70}-C_{90}) α-alkyl branched, and β-hydroxylated fatty acids present mostly as bound esters of arabinogalactan, where they appear primarily as tetramycolylpentaarabinosyl clusters (73, 707). Mycolic acids are also present in the fluid matrix, mainly as trehalose 6,6'-dimycolate (cord factor) and trehalose monomycolate, both of which are distributed on the cell surface (103). The mycolic acids are a major component of the mycobacterial cell wall, making up about 50% by weight of the lipopolysaccharide core of the mAGP complex. They contribute to the thickness of the cell wall, and reducing its permeability. The proportion of mycolic acids containing *trans*-substituents at proximal positions of the meromycolate chain in particular, is an important determinant of fluidity of the mycobacterial cell wall and is also related to the sensitivity of mycobacterial species to hydrophobic antibiotics (1187).

Investigations in the late 1970s indicated that there were four distinct pathways for the formation of the mycolic acids (71). These involve fatty acid chain elongation, desaturation (542, 1159), cyclopropanation of the olefin (332, 1186, 1188), and a Claisen-type condensation (590). Some enzymes of the fatty acid elongation in mycolic acids biosynthesis have been the target of antimycobacterial drugs including the 3-oxo-acyl-

CoA reductase and enoyl-CoA reductase which were inhibited by isoniazid, and the enoyl reductase (InhA) was demonstrated to be a primary target for the latter drug (235).

2.1.7.1.2.4 Lipoarabinomannan (LAM)

Lipoarabinomannan (LAM) is an important mycobacterial cell wall lipid, which is somewhat embedded into the framework of the mAGP, with lipid portion in the cell membrane while the polysaccharide part extends up to the surface of the cell wall. Mycobacterial LAMs are lipoglycans composed of three domains, the mannosyl-phosphatidyl-*myo*-inositol anchor (embedded in the plasma membrane), a polysaccharide backbone, and the capping motifs. There are two forms of LAM, in one form the side chains terminate in arabinose and in the other they terminate in mannose. In the human pathogens, *M. tuberculosis* and *M. leprae* the LAMs are capped with mannoses (154) and are referred to as ManLAMs. In contrast, LAMs isolated from various RGM including *M. smegmatis* are devoid of the mannose cap and small proportion of them terminate with unique inositol phosphate caps, and are termed AraLAMs (154, 841). LAMs exhibit a wide spectrum of immunomodulatory functions. Studies have indicated that LAM suppresses immune responses and mediates the production of macrophage-derived cytokines, thus contributing to pathogenesis and many of the clinical manifestations of pathogenic mycobacteria (46, 147, 515, 978).

2.1.7.1.2.5 Extractable lipids and other cell wall components

The mycobacterial cell wall contains various lipids and related compounds including glycolipids, phenolic glycolipids, glycopeptidolipids, trehalos-containing lipooligosaccharides, sulpholipids (*M. tuberculosis*), and phthiocerol dimycocerosate (*M. bovis* and *M. tuberculosis*) (70, 103, 931). Mycobacterial cell wall also contains a number of waxes (in several slowly growing mycobacteria) and although mycobacteria are not capsulated some of the extractable lipids may exist outside the cell wall proper, and mycosides can give a capsulated appearance to the cell.

The mycobacterial cell wall also contains proteins, for instance the purified cell wall of *M. leprae* contains a 35-kDa major protein (470). Similarly, the cell wall of *M. chelonae* contains a 30-kDa major protein (767), and a 65-kDa porin. The presence of porins is important in that it enables hydrophilic molecules to cross the cell wall.

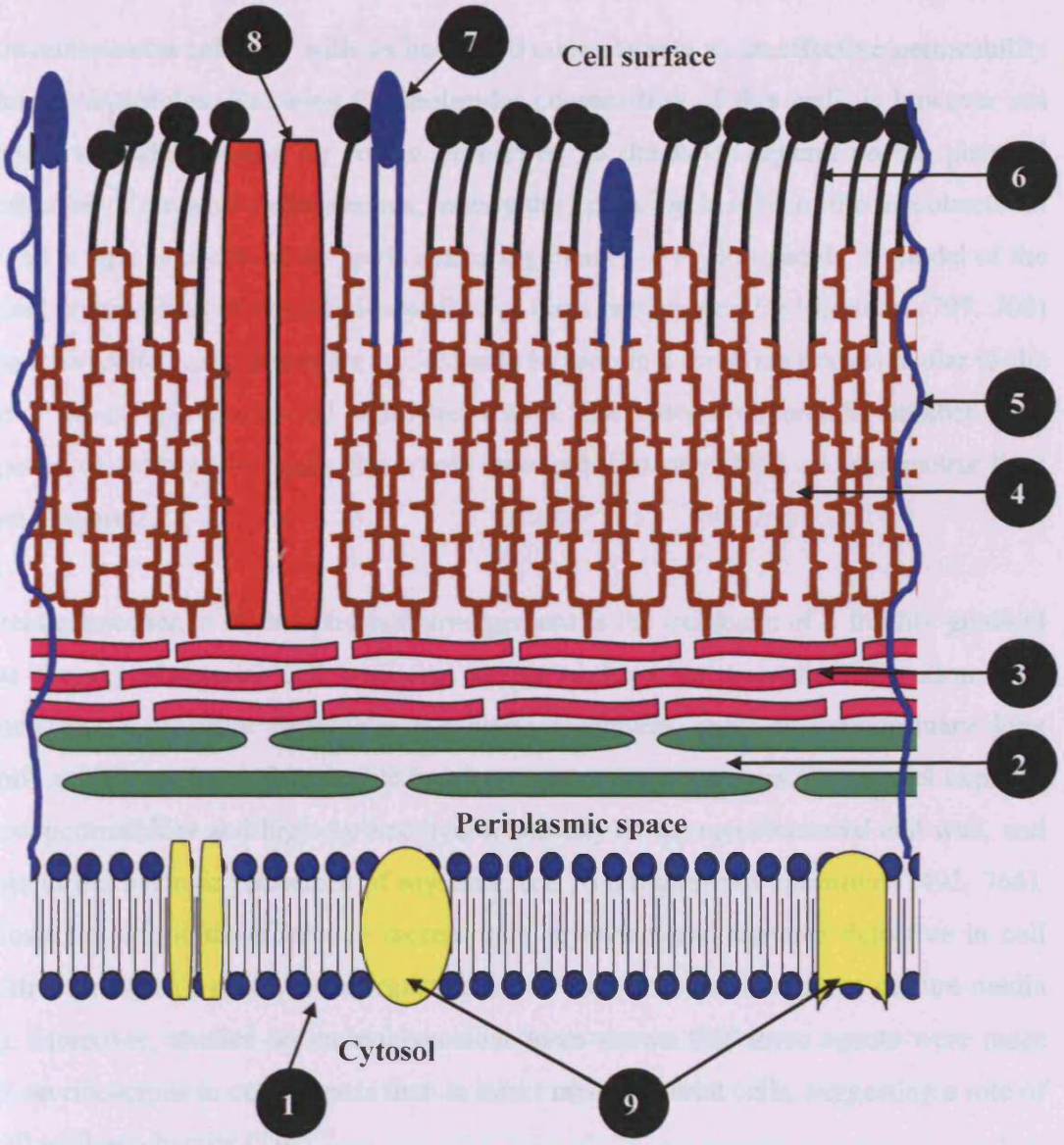


Figure 2.2. Model of the mycobacterial cell envelope. Adapted and modified from Park and Bendelac (793) and Lee *et al.*(591).

1; plasma membrane, 2; peptidoglycan, 3; arabinogalactan, 4; mycolic acids, 5; lipoarabinomannans, 6; glycolipids, 7; free lipids, 8; porin, 9; associated plasma-membrane proteins

2.1.7.2 Cell wall and permeability

The mycobacterial cell wall with its high lipid contents acts as an effective permeability barrier for molecules. Knowing the molecular composition of this wall, is however not sufficient to understanding its barrier properties, as the latter depend on the physical organization of the wall's components, mainly the lipids. Up to 60% of the mycobacterial cell wall weight is occupied by lipids consisting mainly of mycolic acids. A model of the physical organisation of these lipids wall have been put forward by Minnikin (707, 708) in which mycolic acids chains are packed side by side in a direction perpendicular to the plane of the cell surface in cell wall inner leaflet. The latter is covered by another layer composed of extractable lipids, the whole structure thus producing an asymmetric lipid bilayer (Figure 2.2).

A direct consequence of this physical arrangement is the existence of a fluidity gradient across the mycobacterial cell wall that seems to have an opposite orientation. The external region is more fluid than the internal segment with its extraordinary long mycolic acids containing few double bonds or cyclopropane groups. The model explains the low permeability and high surface hydrophobicity of the mycobacterial cell wall, and its role in the intrinsic resistance of mycobacteria to a number of antibiotics (492, 766). For instance, antibiotic efficiency increases in mycobacterial mutants defective in cell wall structure (216), or when detergents such as Tween are added to the culture media (716). Moreover, studies on aminoglycosides have shown that these agents were more active on ribosomes in cell extracts than in intact mycobacterial cells, suggesting a role of the cell wall as a barrier (716).

Although the mycobacterial cell wall is a formidable permeation barrier, production of clinically significant levels of resistance usually requires the involvement of additional resistance mechanisms such as chemical modification of agents, their enzymatic inactivation or degradation and the active efflux of the compounds from the cell (492).

In principle, as it is the case in Gram negative bacteria, the crossing of molecules through the mycobacterial cell wall will be through either porin channels or the lipid bilayer region. Hence it is suggested that there are two pathways for solutes to traverse the mycobacteria wall, depending on their chemistry.

2.1.7.2.1 The hydrophilic pathway

Since hydrophilic solutes can not traverse lipid bilayers, they are predicted to cross the mycobacterial wall through the porin pathways. In this pathway, small hydrophilic antibiotics and nutrients pass through the cell wall via water-filled channels, whereas larger hydrophilic molecules may be unable to cross the wall (248). *M. chelonae*, one of the most drug resistant species of mycobacteria has been used in the study of mycobacterial cell wall permeability. The permeability of this species to the hydrophilic solutes cephalosporins was determined (493), and was shown to be very low, about 1000 times lower than that of the *E. coli* outer membrane, and 10 times lower than that of the very impermeable *P. aeruginosa* outer membrane. The permeation was not affected by temperature and did not increase when more lipophilic cephalosporins were used (493) suggesting that the permeation occurred mainly through aqueous channels.

Trias *et al.* (1074) reported the identification of a 59-kDa cell wall porin in *M. chelonae* that allowed the diffusion of small, hydrophilic solutes. The porin was shown to be a minor protein in the cell wall and had a much lower permeability than porins of equal molecular weight from other bacteria such as *E. coli*. A pore-forming protein with similar properties to that of *M. chelonae* was identified in *M. smegmatis* (1074), and similar porins are probably distributed widely among mycobacteria (103). There are differences in hydrophilic permeability amongst species of mycobacteria. For example, *M. smegmatis* was found to be 10-fold more permeable to β-lactams than *M. chelonae* (1074). This may explain the differences in antibiotic susceptibility among mycobacterial species (Table 2.4).

2.1.7.2.2 The lipophilic pathway

Lipophilic solutes are not the favored solutes for passage through porin channels and thus are likely to diffuse mainly through lipid bilayers. The latter is ordinarily highly permeable to such solutes. However, its permeability is directly correlated with its fluidity (677). Because of the extreme inefficiency of the porin system, the lipophilic pathway may play an important role in solute transport, although this system is slowed down by the low fluidity of the mycolic acid leaflet of the cell wall and its unusual thickness. It is predicted that within each class, the more lipophilic antimicrobial agents would be the more active against mycobacteria. This is supported by the observation that the more lipophilic derivatives of the relatively lipophilic antibacterial agents such as rifampycin, tetracyclines, macrolides, and fluoroquinolones, are more active against mycobacteria (1035, 1109)(390, 856, 1171).

The permeability of the mycobacterial wall to solutes including antibacterial agents is however dependent on other factors and mycobacterial species can have different susceptibilities to the same agent depending on their cell wall components. For instance, *M. chelonae* and *M. fortuitum* are highly resistant to lipophilic inhibitors such as dyes and detergents and have differences in susceptibility to various lipophilic antibiotics. For example, rifampicin, is active against almost all of the clinically relevant species of mycobacteria, except *M. chelonae* and *M. fortuitum* (and *M. aviurn* complex among the slow growers) (1178). Structural variation in the mycolic acids is thought to be the reason for this resistance, as these organisms have a substantial fraction of α-mycolate containing *trans* double bonds at the proximal inner position, which decreases fluidity and permeability of the cell wall (103, 616).

2.1.7.3 Mycobacteria and antibiotics: The molecular basis of resistance

Resistance of mycobacterial species to antibiotics is in part due to intrinsic mechanisms notably their cell envelope permeability barrier. However, resistance can also arise due to mutation and changes in specific targets in the cell. Resistance to rifampicin has been

linked to mutation in the mycobacterial *rpoB* gene, encoding the β-subunit of the RNA polymerase (434, 517, 518, 1146), the *arr* gene, encoding rifampicin ADP ribosyltransferase (10), and the *asnB* gene, encoding a glutamine-dependent asparagine synthetase (865). Resistance to quinolones such as ciprofloxacin was shown to be a result of mutation in the mycobacterial *gyrA* gene (136, 418, 868) encoding the A subunit of DNA gyrase enzyme (331, 1123), whereas resistance to streptomycin was linked to mutations in the 16S rRNA gene (*rrs*) as well as in the *rpsL* and *rpsG* genes encoding ribosomal proteins S12 and S7 respectively (293, 533). Alterations in the *embAB* locus encoding arabinosyltransferases involved in mycobacterial cell wall synthesis, have been reported to cause ethambutol resistance (1001, 1052), while mutations in or complete loss of the *katG* gene, encoding for a mycobacterial catalase-peroxidase, were associated with resistance to isoniazid (1023, 1195). Other genes involved in isoniazid resistance in mycobacteria include the *inhA* and the *ahpC* genes encoding an enoyl reductase, and an alkyl hydroperoxide reductase respectively (236, 433, 1152). The molecular basis of mycobacterial resistance to the above antibiotics and others is summarized in Table 2.5

Table 2.4. Activity of the major chemotherapeutic agents against mycobacteria. Adapted from Jarlier and Nikaido (492).

Agent	*M. tuberculosis*	*M. leprae*	Atypical mycobacteria
Sulfonamides	-	+	-
Penicillin G	-	-	-
Streptomycin	+	-	-
Chloramphenical	-	-	-
Tetracyclines	-	-	-
Erythromycin	-	-	-
Isoniazid	+	-	-
Novobiocin	-	-	-
Vancomycin	-	-	-
β-lactams	-	-	-
Quinolones	-	-	-
Fusidic acid	-	-	-
Rifampicin	+	+	-
Fluoroquinolones	+	+	±

- and + indicate presence and absence, respectively of significant activity.
Minocycline is active against *M. leprae*.
Clarithromycin is active against some atypical species.
Variable depending on species and compound.

Table 2.5. Mycobacterial genes involved in antimicrobial resistance. Modified from Musser (741).

Antimicrobial agent	Species	Gene	Product
Rifampicin (RIF)	*M. tuberculosis, M. leprae, M. africanum, M. avium*	*rpoB*	β-subunit of RNA polymerase
	M. smegmatis	*arr*	Rifampicin ADP ribosyltransferase
	M. smegmatis	*asnB*	Glutamine-dependent asparagine synthetase
Streptomycin (STR)	*M. tuberculosis, M. smegmatis*	*rpsL*	Ribosomal protein S12
	M. tuberculosis	*rrs*	16S rRNA
	M. smegmatis	*rpsG*	Ribosomal protein S7
Isoniazid (INH)	*M. tuberculosis*	*katG*	Catalase-peroxidase
	M. smegmatis	*ahpC*	Alkyl hydroperoxide reductase
INH and ethionamide (ETH)	*M. tuberculosis*	*inhA*	NADH-specific enoyl-acyl carrier protein reductase
		orf1	3-Ketoacyl-acyl carrier protein reductase analog
	M. smegmatis	*ahpC*	Alkyl hydroperoxide reductase
Fluoroquinolones (FQ)	*M. tuberculosis, M. smegmatis*	*gyrA*	DNA gyrase A subunit
Ofloxacin (OFX)	*M. tuberculosis, M. smegmatis*	*gyrA*	DNA gyrase A subunit
Clarithromycin (CLR)	*M. intracellulare, M. chelonae, M. abscessus*	23S rRNA	23S rRNA
Ethambutol (EMB)	*M. tuberculosis*	*embB*	Arabinosyltransferase
Pyrazinamide (PZA)	*M. tuberculosis*	*pncA*	Pyrazinamidase
D-Cycloserine	*M. smegmatis*	*alrA*	D-Alanine racemase
	M. tuberculosis	*ddlA*	D-Alanine–D-alanine ligase

2.1.7.4 Biocides and mycobacteria

Early studies revealed that mycobacteria were more resistant to biocides than other non-sporulating bacteria. In 1971, Croshaw (201) reviewed the response of mycobacteria to biocides and listed ampholytic surfactants (e.g. the 'Tego' compounds), ethylene oxide gas, iodine, alcohols and especially phenolic compounds, notably cresol-soap formulations, as being mycobactericidal. Mycobacteria are generally resistant to acids, alkalis, chlorhexidine, QACs, non-ionic and anionic surface-active agents, heavy metals, and dyes, although many of these agents may inhibit mycobacterial growth without being mycobactericidal (406).

Spaulding *et al.* (1000), Favero (285), and Favero and Bond (286) described three levels of disinfection: High, intermediate, and low. A biocide with high-level activity is lethal to all types of micro-organisms except high numbers of bacterial spores. One with intermediate level of activity inactivates vegetative bacteria including mycobacteria, fungi, and most viruses. Low level activity lacks mycobactericidal effect but non-sporulating bacteria, some fungi and some viruses are inactivated. On the basis of this classification, mycobacteria are considered more resistant to biocides than other non-sporulating bacteria but less resistant than bacterial spores.

2.1.7.4.1 Antimycobacterial agents

Heat is still the most effective way of destroying mycobacteria, which are not especially heat-resistant. However sterilization or disinfection by heat is not always applicable and the use of biocides is more appropriate. The antimycobacterial properties of several of biocides against mycobacteria are summarized in Table 2.6.

2.1.7.4.1.1 Alcohols

Alcohols are usually considered to be mycobactericidal when used at the appropriate concentration. Favero (283) cited alcohol concentrations 70-90% as having an

intermediate level disinfection activity. Ethanol in high concentrations is thought to be an excellent mycobactericidal agent. However, concentrations above 95%, contain too little water to be effective (999). Addition of alcohols to other biocides can increase their germicidal activity, and they are routinely found mixed with other agents such as iodine and formaldehyde in active formulations. Griffiths *et al.* (366) reported that 70% industrial methylated spirits were highly effective against mycobacteria, including *M. chelonae* type strain, glutaraldehyde-resistant *M. chelonae*, *M. fortuitum*, and *M. tuberculosis* H37 Rv. A major disadvantage of alcohols is their relative inactivity in the presence of organic matter. For example, a study by Best *et al.* (75) reported that the lethal effect of 70% ethanol against *M. tuberculosis* was greatly decreased in the presence of sputum.

2.1.7.4.1.2 Phenolic compounds

Phenolics have been considered for many years to be effective against *M. tuberculosis*. Tilley *et al.* (1063) noted their usefulness in this regard, and Richards and Thoen (873) stated that phenolics are mycobactericidal. However, a number of studies reported the relative infectiveness of phenolics against mycobacteria. For instance 3% phenolics have been removed as high-level disinfectants because of their unproven efficacy against *M. tuberculosis* (924), and phenols have been found to be ineffective against *M. smegmatis* in the carrier test (74, 75).

2.1.7.4.1.3 Chlorine compounds

Chlorine compounds have been widely used as disinfectants, and reported to have a wide spectrum of activity against bacteria. Smith (992) reported sodium hypochlorite to be a potent mycobactericidal agent. Other chlorine compounds have been shown to be highly effective against some mycobacteria. These include chlorine dioxide which has been reported to be very active against bacterial spores, *M. tuberculosis*, *M. avium*, and other atypical mycobacteria. Griffiths *et al.* (366) reported that this agent along with a high concentration of a chlorine releasing agent were rapidly mycobactericidal.

2.1.7.4.1.4 Chlorhexidine

Chlorhexidine, another commonly used biocide is considered ineffective against mycobacteria when used at high concentrations (894), however it is mycobacteristatic at low concentrations (107). Chlorhexidine gluconate (4% w/v) was shown to be ineffective against *M. tuberculosis* and produced no more than 2 $\log_{10}$ reduction in viability in suspension tests with or without sputum (75). It is reported however, that mycobacterial susceptibility to chlorhexidines can be increased using other agents that may help increase their cellular penetration. Broadley *et al.* (107) reported that it was possible to enhance the activity of chlorhexidine diacetate, a normally poor mycobactericidal agent, against *M. avium*, *M. bovis* BCG, *M. fortuitum* and *M. phlei* using the antibiotic ethambutol.

2.1.7.4.1.5 Aldehydes

Aldehydes have a wide spectrum of activity, including sporicidal. The mycobactericidal action of aldehydes depends on the individual structure of the agent and the mycobacterial strain it is subjected to. Formaldehyde is generally accepted to have good mycobactericidal activity (890), although there have been a number of reports of the relative ineffectiveness of some formaldehyde formulations against mycobacteria (1089). Formulas containing formaldehyde with other agents, especially alcohols, have been shown to be mycobactericidal (366, 367, 894). Because of its toxicity and corrosiveness, formaldehyde-alcohol has been excluded as a high-level disinfectant (924).

Glutaraldehyde (GTA) has been used as a disinfecting/sterilizing agent for over 30 years (904). Alkaline 2% (v/v) GTA has a broad range of activity and rapid mycobacterial action, and is the most widely used high-level disinfectant for flexible endoscope disinfection (904). The 2% alkaline GTA formulation is now widely regarded as being effective mycobactericidal agent against *M. tuberculosis, M. smegmatis, M. fortuitum,* and *M. terrae* (24, 26, 706, 1089), although variations in resistance of mycobacterial strains to this agent were noted (143, 186). A number of other studies, questioned the

effectiveness of 2% alkalinized GTA against mycobacteria (26). Bergan and Lystad (67) and Rubbo *et al.* (890) reported that the above formulation was not adequately effective against *M. tuberculosis*.

Ortho-phthalaldehyde (OPA) is relatively new aromatic dialdehyde biocide that has been proposed as a possible alternative to GTA for high-level disinfection of endoscopes (1119). Studies have shown that 0.5% (w/v) OPA is effective against a range of mycobacterial species including GTA-resistant *M. chelonae* (311). A number of aldehydes were tested against *M. terrae, M. abscessus*, and both GTA- sensitive and GTA-resistant strains of *M. chelonae*, and it was concluded that 0.5% (w/v) OPA had an acceptable efficacy against mycobacteria (311).

2.1.7.4.1.6 Peroxygens

Peroxygens are generally regarded to have an activity below that of GTA against mycobacteria (105, 207, 448). Hydrogen peroxide (6% v/v) has been reported to be active against *M. bovis*, but had poor performance against *M. tuberculosis*. By contrast the combination of 8% hydrogen peroxide and 0.06% peracetic acid had an excellent tuberculocidal activity (925). Peracetic acid alone is sporicidal (92), which implies that is also mycobactericidal, and it was reported to be effective against GTA-resistant mycobacteria. Lynam *et al.* (631) claimed that Nu-cidex containing 0.35% (v/v) peracetic acid was effective against various mycobacteria including *M. tuberculosis* and GTA-resistant isolates. However, It was reported that GTA-resistant *M. chelonae* isolates were still resistant to 0.035% paracetic acid (1090) but were shown to be susceptible to Cidex PA, containing 1% hydrogen peroxide and 0.08% peracetic acid (1005). Nu-cidex was reported to be effective mycobactericidal agent for use in the disinfection of bronchoscopes after it was shown to effectively eradicate *M. tuberculosis* and *M. chelonae* from bronchoscopes.

2.1.7.4.1.7 Quaternary ammonium compounds (QACs)

QACs are low-level disinfectants that inhibit the growth of some mycobacteria but are not mycobactericidal (299, 305, 914). According to Broadley *et al.* (107) low concentrations of cetylpyridinium chloride inhibited the growth of some mycobacteria including *M. bovis* and *M. avium*. A number of reports however, suggested that some concentrations of QACs can be lethal to mycobacteria, or have similar activity to known mycobactericidal agents. According to Ascenzi *et al.* (19, 20) a QAC had similar tuberculocidal activity to 2% glutaraldehyde, and Holton *et al.* (448) reported that a high concentration of a QAC product was also mycobactericidal. Other workers however, have demonstrated the lack of lethal activity of QACs against mycobacteria (75). Similar to chlorhexidine, QACs activity against mycobacteria can be enhanced using other agents as Broadley *et al.* (107) demonstrated using cetylpyridinium chloride in combination with ethambutol.

2.1.7.4.1.8 Ethylene oxide

Ethylene oxide is a colorless explosive, highly diffusible gas that is considered to be a good tuberculocidal agent and is highly regarded for the used with instruments, especially fiberoptic endoscopes that would be damaged by autoclaving (593, 777). Kereluk *et al.* (534) reported that *M. phlei* cells are less resistant to ethylene oxide than other non-spore forming species tested. Rutala and Weber (928) noted that ethylene oxide sterilization inactivates all micro-organisms including mycobacteria, although bacterial spores were more resistant.

2.1.7.4.1.9 Iodine and iodophors

Iodine and iodophors have historically been considered effective against tubercle bacillus (334, 552). Frobisher and Sommermeyer (317) reported that 1% iodine solution in 10% alcohol was effective against tubercle bacilli contaminating clinical thermometers, although in a later study, Wright and Mundy (1163) found an iodophor solution containing 1.6% iodine to be ineffective the same contaminating organism. Opposing

views have been expressed in regard to the effectiveness of iodine agents against mycobacteria, especially in disinfection of endoscopic equipment. Nelson *et al.* (752) reported that several iodophor compounds, diluted or undiluted, were ineffective against two *M. tuberculosis* strains even after 30 min exposure. Whereas O'Connor and Axon (777) believed that povidine-iodine to be an effective disinfectant for endoscopic equipment. The same agent, at concentration of 0.05% (v/v) was shown to be rapidly mycobactericidal against suspensions of *M. avium*, *M. kansasii* and *M. tuberculosis* (880). Rutala (922) noted that although manufacturer's data demonstrate that commercial iodophors are tuberculocidal, they are no longer considered as high-level disinfectants because of their unproven efficacy against bacterial endospores, *M. tuberculosis* and some fungi.

Table 2.6. Mycobacterial activity of biocides. Adapted from Russell (894) and Hawkey (406).

Compound	Mycobacterial susceptibility	Antimycobacterial activity	Comment
Alcohols	S	C	Susceptibility reduced in presence of organic load
Formaldehyde	S/moderately S	C	Susceptibility increases in combination with alcohol
Glutaraldehyde	S/moderately S/R	C	
Glyoxal	-	C	
Succinaldehyde	-	C	
Chlorhexidine	R	ST	High concentrations not mycobactericidal
Chlorine dioxide	S	C	
Ethylene oxide	S	C	
Hypochlorite	Moderately S/S/ moderately R		
Iodophors	S/moderately S	C	
Ortho-phthalaldehyde	S	-	
Peracetic acid	S	-	Effective against GTA-resistant *M. chelonae*
Peroxygens	S/R	ST/C	Varies with individual compound
Phenols	S	ST/C	Antimycobacterial activity varies with individual compound
QACs	R	ST	Even high concentration normally not mycobactericidal
Superoxidized water	S	-	
β-Propiolactone	-	C	

C, mycobactericidal; ST, mycobacteriostatic; S, susceptible; R, resistant

2.1.7.4.2 Biocides action in mycobacteria

The mechanisms by which mycobacteria are inhibited or killed by biocides are poorly understood. Similarly, it is not clear how biocides are taken up by mycobacterial cells, although some progress have been made due to increased knowledge of the mycobacterial cell wall and the demonstration of the presence of porins in these organisms (section 2.1.7.1) Biocide uptake by mycobacteria is thought to involve adsorption process to the cell wall, which although not yet clearly shown in mycobacteria, it was demonstrated to be the case for many biocides in a number of non-sporulating bacteria (457). The biocides may then cross the cell wall by interaction with the sensitive sites at the cytoplasmic membrane or deeper within the cell (894).

Within the mycobacterial cell, there are a number of potential targets for biocides. These are as follows:

- The cell wall, which unlike the case for chemotherapeutic drugs, it is unlikely to be a major site for biocides. GTA could be an exception, because it is known to interact with the surface of bacterial cells (253).

- The cytoplasmic membrane is a potential target for "membrane-active agents" such as chlorhexidine and cetylpyridinium chloride. These two agents are tuberculostatic at low concentrations, of the same order as those that inhibit other bacteria (106), but not mycobactericidal. This implies that these agents cause membrane damage in mycobacteria but the damage may be insufficient to kill the cells or too small concentration is available to cause cytoplamic damage or both. Their intake into the cell is also thought to be greatly reduced by the cell well (894).

- The cytosol and its components, proteins, enzymes, DNA, and RNA, maybe all be potential targets for biocide activity in mycobacteria. This is proven to be the case in many non-sporulating bacteria (913), although it is yet to be shown in mycobacteria.

2.1.7.4.3 Mycobacterial resistance to biocides

Mycobacteria are generally more resistant to biocides than other non-sporulating bacteria. This difference in susceptibility also exists between mycobacterial species. Development of resistance to some agents has also been reported especially in clinical applications such as endoscope disinfection. Carson *et al.* (143) noted variation in resistance of mycobacterial strains to GTA and formaldehyde and found strains of *M. fortuitum* and *M. chelonae* in commercial distilled water that were very resistant to chlorine. van Klingeren and Pullen (1090) isolated *M. chelonae* from endoscope washers that survived 60 min exposure to alkaline GTA. This resistant strain was also resistant to peracetic acid (631). Mycobacterial resistance to other biocides have been noted, such as *M. chelonae* resistant to 2-3% formaldehyde reported by Hayes *et al.* (408), and triclosan resistance in *M. smegmatis* (683).

Intrinsic resistance, mainly due to the low mycobacterial cell wall permeability and its composition (section 2.1.7) is thought to be an important factor in biocide resistance. The highly hydrophobic mycobacterial cell wall makes it difficult for hydrophilic agents such as hydroxybenzoates to cross the wall in sufficiently high concentrations to achieve mycobactericidal effect. Low concentrations are believed to cross the wall, as MICs of these agents against mycobacteria are generally of the same order as those for other bacteria (894). The relationship between the content of waxy material in the mycobacterial cell wall and resistance of different species to biocides was quoted by Croshaw in 1971 (201). This supports the view expressed by Chargaff *et al.* (153) who reported that resistance to QACs was related to lipid content of the cell wall, since *M. phlei* (with low total lips) was more sensitive than *M. tuberculosis* (with higher cell-lipid content) to these agents. Other work on the QAC, cetylpyridinium chloride, and chlorhexidine diacetate (107), showed that the activity of these two agent against *M. avium* was greatly enhanced in the presence of ethambutol. This suggests that the arabinogalactan component of the cell wall may be involved in the mycobacterial resistance to cetylpyridinium chloride and chlorhexidine diacetate.

Manzoor *et al.* (656) reported that reduce susceptibility to GTA was associated with changes in cell wall polysaccharides and increase in phydrophobicity. GTA-resistant *M. chelonae* were shown to be more hydrophobic than sensitive strains (656). Walsh *et al.* (1120), noted that "decrease in the monosaccharides of arabinogalactan have been associated with an increase in GTA resistance, suggesting that the arabinogalactan and arabinomannan portions of the cell wall could be involved in the resistance mechanism, possibly hindering the penetration of GTA".

One of the few examples of the identification of a mycobacterial genes involved in biocide resistance was the elucidation of triclosan resistance in *M. smegmatis*. McMurry *et al.* (683) selected for triclosan resistance in *M. smegmatis* mc^2155. Three mutants which had over 4 times the triclosan MIC of the wild type were examined, and were found to have different mutations in their *inhA* gene. The InhA is an enoyl reductase involved in fatty acid synthesis that is 35% identical to the *E. coli* FabI. Two of the mutants did also express some isoniazid resistance, which was not surprising as the enoyl reductase is a target for this drug in *M. smegmatis*.

Another possible mechanism for biocide resistance in mycobacteria is the natural transport system and expression of efflux pumps in these organisms. Several mycobacterial efflux pumps have been characterised (225, 605, 1097), and the role of some in mycobacterial intrinsic and acquired drug resistance have been reported (939). Banerjee *et al.* (41) reported that overexpression of the phosphate-specific transporter (Pst) in *M. smegmatis* selected for ciprofloxacin resistance. They concluded that the Pst is a natural membrane transport system involved in efflux-mediated drug resistance in *M. smegmatis*. In another study Stephan *et al.* (1014) reported that a *M. smegmatis* mutant lacking the major porin MspA expressed increase resistance to a number of drugs including ampicillin, cephaloridine, vancomycin, erythromycin and rifampin.

Efflux pumps have been studied in *M. smegmatis* in relation to drug resistance. Takiff *et al.* (1045) showed that the LfrA, a major facilitator superfamily transporter, confered resistance to ethidium bromide, acriflavine, and some fluoroquinolones when

overexpressed from a multicopy plasmid. Disruption of the *lfrA* gene rendered the mutant more susceptible to ethidium bromide, acriflavine, ciprofloxacin, doxorubicin, and rhodamine (939). These results were also confirmed by Li *et al.* (606), who used *M. smegmatis* with mutations in *lfrA* as well as the homologues of *M. tuberculosis* Rv1145, Rv1146, Rv1877, Rv2846c (*efpA*), and Rv3065 (*mmr* and *emrE*), all expressed in the organism and encoding putative drug efflux pumps. Mutants in the *lfrA* gene, the *mmr* homologue and the *efpA* homologue rendered the mutants more susceptible to multiple drugs such as fluoroquinolones, ethidium bromide, and acriflavine. Although the involvement of efflux pumps in biocide resistance in mycobacteria is yet to be clarified, it is possible that this mechanism is a significant factor

2.2 AIMS

The main aim of this study was to determine the molecular basis of biocide susceptibility and resistance in mycobacteria. This was to be achieved by:

a) Examining the susceptibility of mycobacterial strains (*M. smegmatis* mc^2155 as a model strain, and *M. chelonae* and *M. abscessus* type strains) to biocides (*ortho*-phthalaldehyde, triclosan, cetylpyridinium chloride, and chlorhexidine diacetate) and antibiotics.

b) Using transposon mutagenesis to generate random *M. smegmatis* mc^2155 mutants and subsequently screening these mutants to isolate biocide resistant/sensitive derivatives.

c) Evaluating the level of susceptibility of the isolated mutants to the four biocides described above using agar and broth MICs and lethality tests.

d) Using the DNA sequence flanking each transposon mutant to identify the disrupted gene by correlation to the *M. smegmatis* mc^2155 genome sequence, and confirming the result by complementation.

e) Comparing the antibiotic profiles of the wild type *M. smegmatis* mc^2155 with those of the biocide mutants to determine any cross-susceptibility between biocides and antibiotics.

The overall hypothesis was that gene transposon mutagenesis would enable the molecular basis of biocide resistance or susceptibility in *M. smegmatis* mc^2155 to be determined. The complete experimental strategy to be followed is outlined schematically in Figure 2.3.

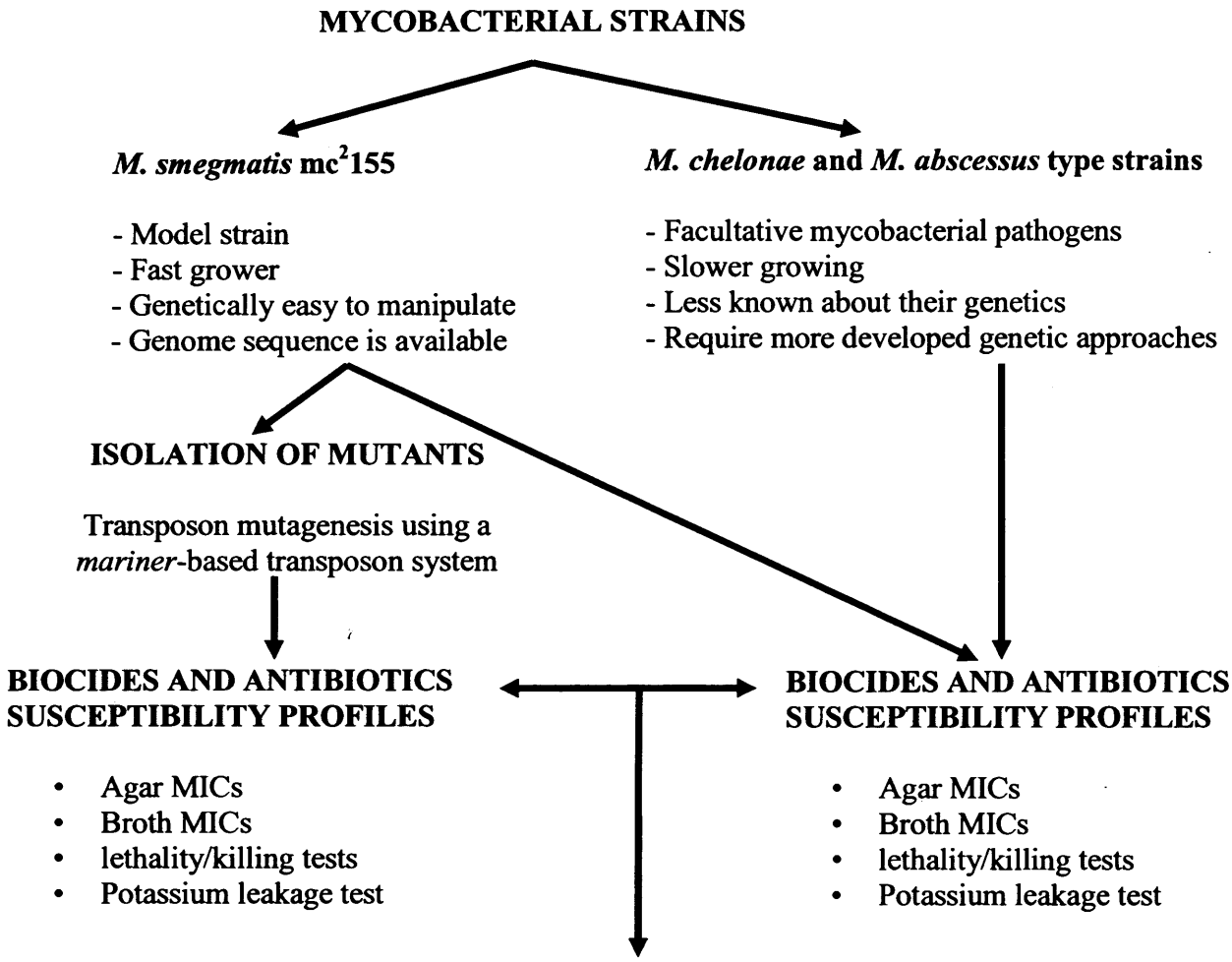


Figure 2.3. Experimental strategy used in the current study.

The experimental strategy used in this study was divided into three major sections: Firstly, biocide and antibiotics susceptibility profiles were to be determined for the model organism *M. smegmatis* mc²155, and for *M. chelonae* and *M. abscessus* type strains. This was to be achieved by evaluating agar and broth MICs, the killing effect of biocides, the amount of potassium leaked from biocide-treated cells, and determining the agar MICs for selected antibiotics. Secondly, transposon mutagenesis using a *mariner*-based transposon system was to be used to generate and isolate biocide mutants of *M. smegmatis* mc²155. The biocide susceptibility profiles of these mutants were to be determined as for the wild type mc²155. Agar MICs of selected antibiotics were also to be determined for the biocide mutants to find out whether change in susceptibility to biocides had an effect on antibiotic sensitivity. Thirdly, the molecular basis of the phenotypic change in the biocide mutants was to be determined by identification of the transposon-disrupted gene in the mutant's genome using molecular techniques including PCR, DNA sequencing and bioinformatics. The results were to be confirmed by complementation analysis.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and plasmids

Bacteria strains and plasmids used in this study are described in Table 2.7.

Table 2.7. Bacterial strains and plasmids.

Strain or plasmid	Reference/ catalogue number	Comments
Strains		
M. smegmatis mc^2155	(993)	Efficient plasmid transformation mutant
M. smegmatis HS42	(789)	
M. chelonae	NCTC 946	Type strain
M. abscessus	ATCC 19977	Type strain
E. coli JM109	(324)	Containing plasmid pM272B
Plasmids		
pM272B	(324)	pPR27 containing *mariner* transposon cassette: 5' and 3' inverted repeats of *Mos1* surrounding the KAN^r cassette of Tn*903* (from *MosK*ΔCD (1066)) and the *Mos1* transposase (*mTpase* from pET3a-Tpase (1066), which is under the control of the mycobacterial *groEL* promoter (*groELp* from pSMT3 (325)) (Figure 2.5).

2.3.2 Media and growth conditions

Mycobacterial cultures were grown on Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco). When required the following media were also used: Sauton minimal medium (1000 ml of distilled water containing: 0.5g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 2g citric acid, 0.05g ferric ammonium citrate, 60 ml glycerol, 4g asparagine, and 0.1 ml of a 1% $ZnSO_4$ solution), Lemco broth (1000 ml distilled water containing:10g Bacto-peptone, 5g Bacto-Lab Lemco powder, and 5g NaCl at pH of 7.2), and Tryptone Soya Broth (TSB) (Difco). Unless mentioned otherwise, all mycobacterial strains were cultured with gentle shaking at the incubation parameters described in Table 2.8. Growth on solid agar was achieved using either, Middlebrook 7H11 agar (Difco), supplemented with 0.5% glycerol and 10% OADC, or Tryptone Soya Agar (TSA) (Difco). When required, both solid and liquid media used to grow mycobacterial strains were supplied with the appropriate concentration of kanamycin.

E. coli JM109 strains used for pM272B plasmid extraction (section 2.3.11.2) and transformation experiments (section 2.3.15.1) were grown in Luria-Bertani (LB) (Difco) broth (936) or on TSA at 37°C. When required, LB broth and TSA agar were supplied with 25 µg/ml kanamycin. Freezer stocks of bacterial strains were prepared by addition of 8% v/v Dimethyl sulfoxide (DMSO) (Fisher Scientific) to a fresh suspension of bacteria. Strains were stored at -80^{0}C.

Optimal growth conditions and growth characteristics of mycobacterial strains in broth were determined by growth curves monitoring the change in optical density (OD) readings at 630 nm of the cultures over time. OD values were generated using an automated plate reader (section 2.3.5). Optimal growth conditions for mycobacterial strains were determined using a range of media (Lemco media, TSB, Sauton minimal media, and 7H9 broth), and growth temperatures (30^{0}C and 37^{0}C).

Table 2.8. Growth conditions of mycobacterial organisms.

Organism	Period of incubation (days)	Temperature of incubation (^{0}C)
M. smegmatis mc^{2}155	2	37
M. smegmatis HS42	2	37
M. chelonae	4	30
M. abscessus	4	37

2.3.3 Preparation of mycobacterial cultures

Before being used for testing, all mycobacterial cultures were prepared as follows:
Strains were grown from a single isolated colony vigorously dispersed in the appropriate liquid medium and grown at the appropriate growth parameters (Table 2.8). Cultures were then passed at least 20 times through a 26-gauge needle to separate cells and avoid clumping. The resulting suspensions were then used for testing as appropriate.

2.3.4 Viable cell count

The number of colony forming units (cfu) per ml of bacterial cell suspension was calculated using either drop counting method or spreading method. In the drop counting method, bacterial suspensions (section 2.3.3) were serially diluted in either 0.85% saline or the broth medium in which bacteria had been grown. A series of 10-fold dilutions were performed by adding a 100 µl of the bacterial suspension to 900 µl of saline or broth. The dilution series were mixed using a vortex mixer and 10 µl of each dilution were dropped in triplicate onto the surface of an agar plate. Plates were left to dry at room temperature for 15 to 20 min before incubation at the appropriate temperature until the colonies could be counted. The number of colonies present in a countable dilution was used to calculate the viable cell count expressed as cfu/ml suspension.

In the spreading method, dilution series of the bacterial suspension to be enumerated were produced as mentioned above. A 100 µl of each dilution was dispensed on the surface of a well dried agar plate and spread evenly over the agar surface using a sterile glass rod. The plates were incubated inverted at the appropriate temperature. The dilution plates containing between 30 and 300 colonies was used to calculate the viable count.

2.3.5 Measurement of optical density

Optical density (OD) of bacterial suspensions were measured at a wavelength of 630 nm using an automated plate reader (MRX^{TC} revelation, DYNEX Technologies), with the

bacterial growth held in sterile 96-well plates. When ODs of cultures were measured without using the automated plate reader, they were transferred into 1 ml disposable plastic cuvettes (Fisher) and the OD measured using a Helios α UV-visible spectrophotometer (Spectronic Unicam) at 630 nm wavelength. Absorbance was always measured against an appropriate blank.

2.3.6 Relationship between OD and viable counts

Experiments were also performed to determine the relationship between the OD measurements and the number of cfu present for the corresponding ODs of bacterial suspensions. The OD reading at 630 nm of bacterial culture grown overnight in appropriate broth media and prepared as described in (section 2.3.3) was measured and viable count of the number of cfu present was determined as described above. The original suspension was then diluted in sterile deionised water to a new OD and the number of cfu present at the new OD was also determined. This process was repeated until a standard curve showing the relationship between OD versus $\log_{10}$ cfu/ml could be obtained. Cell densities of liquid cultures were then determined by comparing the OD value of the culture suspension at 630 nm wavelength to the standard curve determined above.

2.3.7 Biocides and biocides solutions

The following biocides were used in this study: triclosan (Ciba Speciality Chemicals, Grenzach, Germany), chlorhexidine diacetate (CHX) and cetylpyridinium chloride (CPC) (ICN Biomedicals Inc, Ohio, USA), and *ortho*-phthalaldehyde (OPA) (Advanced Sterilization Products division, Johnson & Johnson, USA). Biocide stock solutions were made up fresh on the day of use in deionised water and filtered sterilised, with exception of triclosan and *ortho*-phthalaldehyde which were prepared in DMSO. The stock solutions were then diluted to the required concentration for use. The final concentration of DMSO added to the medium was not lethal to the organism being investigated.

2.3.8 Antibiotics

In this study the antibiotic used was: Kanamycin (KAN), (Sigma Aldrich, Gillingham, UK). Stock solutions of the antibiotic were prepared in deionised water, filter sterilized, and stored at -20^{0}C. E-test strips (AB Biodisk, Bio-Stat Ltd) containing amikacin (AMK), azithromycin (AZM), chloramphenicol (CHL), ciprofloxacin (CIP), tobramycin (TOB), trimethoprim/sulfamethoxazole (SXT), meropenem (MEM), ceftazidime (CAZ), imipenem (IPM), and piperacillin (PIP) were also used.

2.3.9 Biocides MICs determination

Minimal inhibitory concentrations (MICs) of biocides were determined in both liquid and solid media. Biocide solutions were made up fresh on the day of use as described in section 2.3.7, and diluted to the required concentration of use. For MIC determination in liquids, 96-well plates were filled with growth medium containing the appropriate concentration of biocide tested. A culture of the organism to be tested (section 2.3.3) was diluted to an OD of 0.5 ($\approx 10^8$ cfu/ml) and used to inoculate the 96-well plates using a multi-inoculator, the plates were then incubated at the appropriate growth parameters (Table 2.8). Inhibition of growth was determined by reading the OD of cultures at 630 nm using an automated plate reader and comparing the results against controls grown in the absence of the biocide. Broth MICs were taken as the minimal concentrations of biocides that had mean OD values which showed no statistically significant difference from the mean value of the blank after growth at the appropriate growth parameters (Table 2.8). MIC of DMSO for the tested organisms was also determined in broth as described above to evaluate DMSO toxicity on the organisms. Agar MICs were determined using 7H11 agar plates containing the appropriate concentrations of biocides, inoculated with mycobacterial cultures of OD_{630nm} of 0.5, and observing inhibition of growth after incubation at the appropriate growth parameters (Table 2.8).

2.3.10 Antibiotic MICs

Antibiotic MICs were determined using E-test on 7H11 agar according to the manufacturer's instructions (AB Biodisk, Bio-Stat Ltd). Briefly, organisms to be tested were grown in 7H9 broth as described in section 2.3.3. Cells were then washed twice by centrifugation at 3000 rpm for 10 min to remove culture media which might interact with the test antibiotic, and passed at least 20 times through a 26-gauge needle to separate cells and avoid clumping. An inoculum of approximately 10^8 cfu/ml was obtained by serial dilution in 0.85% saline. A sterile swab was immersed into the cell suspension, excess culture was removed, and the culture was then spread evenly in three directions across a well dried 7H11 agar plate. Plates were then left to dry for 15 min at room temperature, prior to the application of the E-test strip onto the agar surface. Plates were then incubated at the appropriate growth parameters (Table 2.8) and analysed. Determination of the antibiotics MICs by E-test were performed at least in duplicate, and the MICs were interpreted at the point of intersection between the inhibition zone and the E-test strip (Figure 2.4).

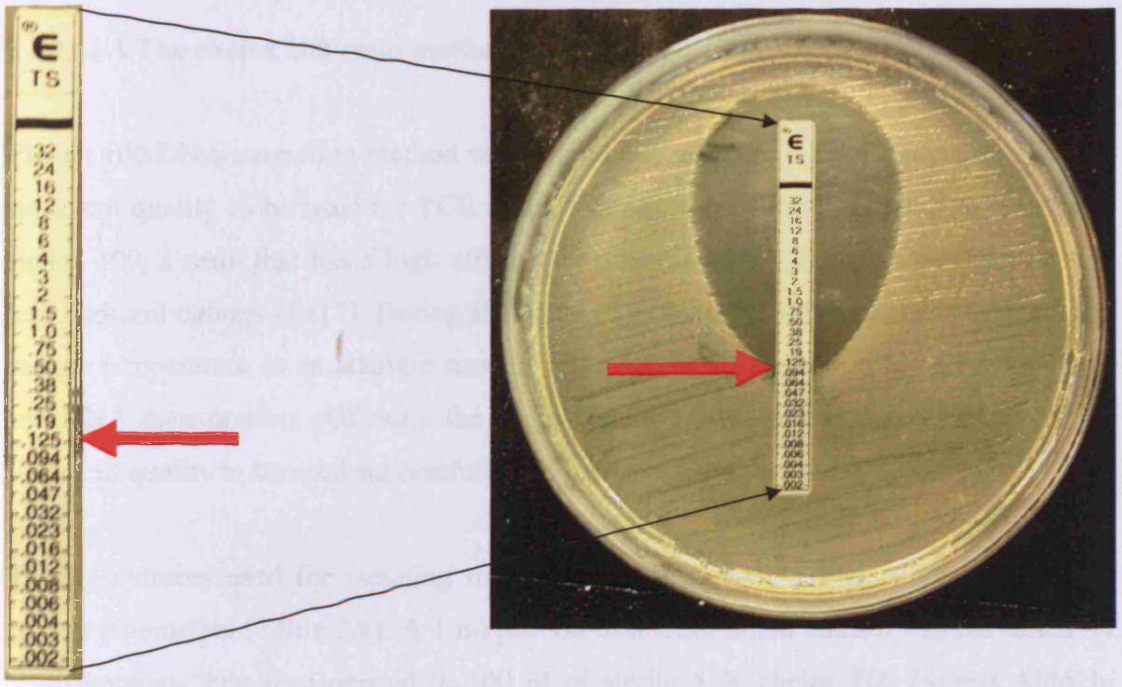


Figure 2.4. Antibiotic susceptibility determination using E-test strips.

Antibiotic MICs for bacteria were determined using E-test strips. The figure showes *Serratia marcescens* Db11 grown on TSA agar plate with a trimethoprim/sulfamethoxazole (1/19) E-test strip. The Red arrows point to the antibiotic MIC value in µg/ml, which represents the point of intersection between the inhibition zone and the E-test strip.

2.3.11 Nucleic acid extraction and manipulation

2.3.11.1 Genomic DNA extraction

Three methods were used for DNA extraction from bacteria:

2.3.11.1.1 The chelex 100 resin method

Chelex 100 DNA extraction method was a rapid and efficient way of extracting DNA of sufficient quality to be used for PCR amplification. The method is based on the use of chelex 100, a resin that has a high affinity for polyvalent (eg. Copper) over monovalent (eg. Sodium) cations (1117). During the chelex DNA extraction steps, cells are exposed to high temperature in an alkaline suspension, resulting in disruption of cell membranes and DNA denaturation. Although the chelex method produce denatured DNA, it is of sufficient quality to be used successfully in PCR amplification (1117).

Briefly cultures used for isolating the genomic DNA were grown at the appropriate growth parameters (Table 2.8). A 1 ml portion of a fresh liquid culture was harvested by centrifugation, then resuspended in 100 µl of sterile 5 % chelex 100 (Sigma Aldrich, Gillingham, UK). The sample was then boiled for 5 min, and immediately placed on ice for a further 5 min. The sample was subjected to a further round of boiling and chilling on ice, then centrifuged at 13000 rpm for 5 min. The resulting supernatant containing the DNA was then transferred to a clean micro-tube and used for PCR or stored at -20^{0}C for further use.

2.3.11.1.2 The boiling method

DNA intended for PCR analysis was also generated using a boiling method. It is a faster and more convenient way of extracting DNA for PCR analysis. In the boiling method, suspensions of the cells were made in 100 µl TE buffer (10 mM Tris-Cl pH 8, 1 mM

EDTA pH 8), and then boiled for 10 min. After centrifugation at 13000 rpm for 5 min, the supernatant containing the DNA was used for PCR.

2.3.11.1.3 The guanidinium chloride method

The guanidinium chloride method was used to extract the total genomic DNA from mycobacterial stains. The method adapted from that described by Katoch and Cox (520) is based on the use of guanidinuim chloride for DNA extraction. Briefly, cultures of mycobacteria grown to their exponential phase in the appropriate media, were harvested by centrifugation at 3500 rpm for 10 min. The pellets were resuspended in about 2 ml of SET buffer (0.3 M sucrose, 50 mM Tris-CL pH 8, 10 mM EDTA) containing 2 mg/ml lysozyme and lipase. The suspensions were then incubated at 37^0C with shaking for 2 hours.

The weakened cells were then pelleted by centrifugation and resuspended in SET buffer to give a "smooth paste" of unclumped cells. Mycobacteria were lysed by the addition of 2 ml of guanidinium chloride buffer (6 M guanidinium chloride, 0.1% Tween 80, 10 mM EDTA, 1 mM 2-mecaptoethanol). Once the lysis of cells was completed (the suspension of cells cleared and became viscous), the supernatant solution of DNA was extracted by addition of an equal volume of chloroform, followed by mixing and centrifugation. DNA was precipitated by the addition of 3 volumes of 100% ethanol to the lysis solutions and agitating them slowly until the two phases were mixed completely. The precipitated DNA formed single clot which was easily removed using a pipette. DNA was then washed twice with 70% ethanol, dried under vacuum and dissolved in 100 µl of TE buffer containing 0.5 µg/ml RNAase (Sigma-Aldrich Ltd, UK). Dissolved DNA was visualized by agarose gel electrophoresis (section 2.3.12) and stored at 4^0C until required.

2.3.11.2 Plasmid DNA extraction

Plasmid DNA extraction from *E. coli* JM109 was carried out using the standard alkaline lysis method (936). Briefly 2-5 ml cultures of *E. coli* JM109 with the appropriate

antibiotic selection for the plasmid were grown overnight at 37^0C with gentle shaking. A 1.5 ml portion of the culture was harvested in a microcentrifuge tube at 13000 rpm for 1 min. After discarding the supernatant, the cells were evenly resuspended in 100 µl TE, and a 200 µl of a freshly made alkaline-SDS (0.2 M NaOH, 1% SDS) solution was added. The content of the microcentrifuge was immediately mixed by gentle inversion and left to stand at room temperature for exactly 5 min. A 150 µl of 3 M sodium acetate pH 5.5, was then added and mixed with the tube content, which was left to stand on ice for a further 10 min. The mix was then centrifuged at 13000 rpm for 3 min, and about 500 µl of the clear supernatant was removed into a clean tube. A 500 µl of chloroform was then mixed with the contents of the tube, which was then centrifuged at 13000 rpm for another 3 min. The upper aqueous phase that formed on the mix was then transferred into a clean tube, taking care not to transfer any of the debris that lies at the interface. DNA precipitation was then performed by mixing 0.7 volumes of isopropanol (propan-2-ol) with the tube content, which was then left to stand at room temperature for 5 min, before centrifugation at 13000 rpm for 20 min. The supernatant was discarded and the pellet washed with 500 µl of 70% ethanol, before another round of centrifugation at 13000 rpm for 1min. Ethanol was then discarded and the pellet dried under vacuum for 20 min. The resulting plasmid DNA was then dissolved in 50 µl of either TE buffer or sterile polished water, both containing 0.5 µg/ml RNAase. Plasmid DNA was stored at 4^0C until required.

2.3.11.3 Quantification of nucleic acid

In this study, nucleic acid concentration in solutions was quantified using the Gene Quant system (Pharmacia Ltd, UK) at Cardiff University's Molecular Biology Service Unit according to the manufacture's instruction. The system uses the A_{260} / A_{280} absorbance ratios of nucleic acid solutions to calculate the concentration.

2.3.11.4 Digestion of chromosomal and plasmid DNA

Digestion of both genomic and plasmid DNA was performed according to the manufacturer's instructions (Promega Corporation Inc, Southampton, UK). Unless otherwise stated, DNA digestion was made in a 20 µl volume containing the appropriate enzyme, buffer, and 3 to 12 µg of DNA. Reaction was mixed then incubated at the optimum temperature for up to 12 h. When required, enzyme inactivation was performed by heat denaturation at the appropriate temperature. Digested DNA was separated and analyzed by agarose gel electrophoresis (section 2.3.12).

2.3.12 Agarose gel electrophoresis

Standard agarose gel electrophoresis was carried out to separate DNA fragments using agarose (Invitrogen Life Technologies) prepared in 1X Tris-EDTA (TBE) buffer (0.04 M Boric acid, 0.04 M Tris, 1 mM EDTA, pH 8) (936). Samples of DNA (5 to 20 µl) were routinely added to appropriate volumes of DNA loading dye (Sigma-Aldrich Ltd, Poole, UK), and loaded into the gels for analysis. For molecular size comparison, the 1Kb+ DNA ladder (Gibco BRL Life Technologies, UK) was used. Unless otherwise stated, electrophoresis was carried out in 1 to 1.5% agarose gels at 80 to 100 volts (Bio-rad power PAC 300). After the dye has travelled at least 75% of the gel length, gels were stained by immersion in 1X TBE buffer containing 0.5 µg/ml ethidium bromide for 30 to 60 min as appropriate. DNA was analysed on a UV transilluminator and photographed using Gene Genius Bioimaging system (SynGene, Syntopics Ltd, Cambridge, UK).

2.3.13 Polymerase chain reaction (PCR)

PCR primers and target DNA for amplification are listed in Table 2.9. Primers were ordered from MWG Ltd Biotech (Milton Keynes, UK). The primers were dissolved to 100 pmol/µl in accordance with the manufacturer's instructions, then checked on an agarose gel. Regions of DNA were amplified using the polymerase chain reaction (933) as described by Sambrook *et al* (936). Unless otherwise stated, PCR amplifications were

performed using Promega PCR kit reagents (Promega corporation Inc, Southampton, UK), and in a standard 25 µl reaction mixture containing PCR buffer (1X manifacturer's stock), 1.5 mM $MgCl_2$, 200 µM of each of the deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), 1 unit of Taq polymerase, 5 to 10 pmol of each forward and reverse primers and roughly 10-50 ng/µl of DNA template. Both a positive (pM272B DNA) and negative (sterile polished water) control were included to confirm amplification of the correct DNA sequence.

DNA amplification was carried out with a MJ Research PTC-200 thermal cycler using block control and a heated lid. Unless otherwise stated, the reaction program consisted of initial 5 to 10 min DNA denaturation at 94^0C as appropriate, followed by 30 cycles of 30 sec denaturation at 94^0C, 30 sec of primer annealing at 50^0C, and a 1.5 min elongation step at 72^0C. The process was terminated by a final elongation stage for 5 min at 72^0C.

Table 2.9. Details of the PCR primers and DNA targets used in this study.

Primer	Primer sequence 5'-3'	Annealing temperature (^{0}C)	Product size (bp)	Specificity / use	Reference / source
KN-15 KN-23	GAGGCAGTTCCATAGGATGG TCAGGTGCGACAATCTATCG	55	620	Amplify the kanamycin resistance (KAN^r) gene.	(324)
SACB-15 SACB-23	ACCCATCACATATACCTGCC ATCGTTAGACGAAATGCCGT	55	1422	Amplify the levansucrase gene (*sacB*).	(324)

2.3.14 DNA hybridization

DNA hybridization was used to check for single random insertion of the *mariner* transposon into the *M. smegmatis* genome. The KAN^r gene of the transposon was used as a probe (Table 2.9).

2.3.14.1 Probe synthesis

The non-radioactively, digoxigenin (DIG), labeled probe was constructed as follows. The KAN^r gene was amplified by PCR (611) as previously described in section 2.3.13, using the pM272B DNA as a template, with the modification of using DIG-labelled dNTPs (Roche Diagnostics Ltd, Lewes, UK) in place of the standard dNTPs. Successful incorporation of the DIG-dNTPs into the PCR products was checked using standard agarose gel electrophoresis (section 2.3.12). DIG-labeled PCR products showed an increased molecular weight when compared to the same non-labelled PCR products. Suitable probe concentration for use in hybridization experiment was estimated by spotting 1 µl of the probe in a dilution series onto a positively charged nylon membrane (Roche Diagnostics Ltd, Lewes, UK). DNA was fixed on the membrane by exposure to short wavelength UV light (320 nm wavelength) for 3 min and probe was detected as described in section 2.3.14.4. The probe dilution that gave a strong hybridization single with minimal background was used. Probes were stored at -20^0C for future use.

2.3.14.2 Transfer of DNA to a nylon membrane

Genomic DNA was digested with *Pst*I, *Sal*I and *Hind*III restriction enzymes, none of which cut within the KAN^r gene sequence of the *mariner* transposon, and separated by standard agarose gel electrophoresis (section 2.3.12). The digested DNA was then transferred to a positively charged nylon membrane (Roche Diagnostics Ltd, Lewes, UK) as described by Sambrook *et al.* (936). Briefly, following electrophoresis, gel was cut to size using a clean, sharpe scalpel and washed in 1X TE buffer. DNA was denatured by gentle agitation of the gel in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 min

at room temperature. Denaturation solution was then replaced and agitation continued for a further 15 min. DNA was transferred to a positively charged nylon membrane using Quick draw paper (Sigma-Aldrich Ltd, Pool, UK) as follows: eight sheets of Quick draw paper were cut to the size of the gel, and four sheets were socked in denaturation solution and put on top of each other in a clean plastic try. The agarose gel was positioned DNA faced down on top of the soaked Quick draw sheets, and a positively changed nylon membrane (Roche Diagnostics Ltd, Lewes, UK) was placed directly on top of the gel. One sheet of Quick draw paper soaked in denaturation solution was placed on top of the nylon membrane, followed by three additional dry sheets. Denaturation solution was added to the bottom of the plastic try and a suitable weight was placed on top of the Quick draw paper sandwich. The resulting squash blot was left overnight at room temperature. After DNA transfer, the membrane was soaked in neutralizing solution (3 M NaCl, 0.5 M Tris-HCl, pH 7.5) for 5 min, then washed in 2X Sodium chloride-Sodium citrate buffer (SSC; 0.15 M NaCl, 0.015 M sodium citrate). DNA was fixed to the membrane by 3 min exposure to UV light (320 nm wavelength). Membrane was air dried, wrapped in cling film and stored at room temperature.

2.3.14.3 Southern hybridization

Membrane containing genomic DNA was transferred to hybridization tube with DNA side facing the tube's interior. Pre-hybridization of the membrane, where non-specific nucleic acid binding sites on the membrane were blocked, was performed at 50^0C with rotary mixing in a hybridization oven (Bibby Stuart Scientific hybridization oven) for 1 h in 20 ml of DIG Easy Hyb buffer (Roche Diagnostics Ltd, Lewes, UK). The DIG-labeled probe was heat-denatured by boiling for 5 min, and 10 µl of the latter was mixed with 500 µl of DIG Easy Hyb buffer and added to the hybridization tube. Hybridization was carried out overnight at 50^0C with rotary mixing. The removal of unbound or non-specific bound probe was achieved by high stringency (at 68^0C) post-hybridization washes. These consisted of two 15 min washes with each of the following washing solutions: 2X SSC-0.1% SDS, 0.5X SSC-0.1% SDS and 0.1X SSC-0.1% SDS,

respectively. All washing solutions were preheated to the post-hybridization temperature before use.

2.3.14.4 Detection of the probe

Detection of the hybridization signal was carried out at room temperature on a rocking platform. Membrane was equilibrated in washing buffer (0.1 M Maleic acid, 0.5 M NaCl, 0.3% Tween 20), then washed in blocking solution (5% skimmed milk dissolved in Maleic acid buffer [0.1 M Maleic acid, 0.1 M NaCl, pH 7.8]) for 30 min, and in antibody solution (Anti-DIG-AP fragment [Roche Diagnostics Ltd, Lewes, UK] diluted 1:10000 in blocking solution) for a further 30 min. The membrane was then washed twice in washing buffer for 15 min each wash, followed by transfer into a plastic try and soaking in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 min. The next stage involved the construction of a development folder and chemiluminescent detection of the DIG-labelled probe. The membrane, with DNA side up, was placed onto a clean sheet of acetate and 2 ml of a 1:100 dilution of disodium 3(4-methoxispiro[1,2,dioxetane-3,2-{5'Chloro}Tricyclo.3.1.1^{3,7}]decan)-4-yl phenyl phosphate (CSPD [Roche Diagnostics Ltd, Lewes, UK]) prepared in detection buffer was distributed all over the membrane surface. Another sheet of acetate was placed on top of the membrane, making sure that the CSPD solution covered the entire membrane surface and avoiding any air bubbles. The resulting development folder was sealed in cling film with DNA side clear. The folder was incubated for 15 min at room temperature for the light producing reaction to reach equilibrium. Hybridization bands were developed by exposing the membrane to autoradiography film (Sigma-Aldrich Ltd, Pool, UK), in an exposure cassette, in the dark, at room temperature for up to 12 h until a clear hybridization signal was obtained. Film was processes using the Kodak GBX developer and fixer solutions according to the manufacturer's instructions (Sigma-Aldrich Ltd, Pool, UK), and left to dry at room temperature before analysis.

2.3.15 Mutant generation and screening

2.3.15.1 Electrotransformation and transposon mutagenesis

2.3.15.1.1 Electroporation

Electrotransformation of *M. smegmatis* mc^2155 cultures with pM272B vector was conducted as follows. A fresh 3 ml of an overnight culture of *M. smegmatis* mc^2155 was diluted 50 times in 7H9 broth (1 ml of culture into 49 ml of broth) and grown overnight at 37^0C with shaking. At this stage, the cells in their mid-log phase of growth were harvested at 3500 rpm for 10 min, washed in 20 ml wash buffer containing 10% glycerol and 0.05% Tween 80, then centrifuged at 3500 rpm for a further 10 min. The resulting pellet was then resuspended in 4 ml buffer containing 10% glycerol and viable count (section 2.3.4) was performed on the suspension. Competent bacteria (100 µl) were mixed with 5 µl of plasmid pM272B, incubated for 10 min to facilitate DNA adsorption, transferred into a 0.2-cm-gap electroporation cuvette, and subjected to a pulse of 2.5 kV, 25 µFD, and 400 Ω using an electroporation apparatus (Bio-Rad Laboratories Ltd, Hertfordshire, UK).

Electroporated bacteria were incubated in 1 ml of 7H9 broth at 30°C for 2 h, repeatedly passed through a 26-gauge needle, before serial dilutions of the cultures were plated onto 7H11 agar containing 25 µg/ml KAN (positive selection). As a negative control to estimate the level of spontaneous resistance to KAN, 100 µl of competent cells lacking plasmid (pM272B-free), were plated onto 7H11 agar containing KAN. Transformants were observed after 4 to 5 days of incubation at 30^0C. Transformation frequency was calculated as a proportion of transformants cfu per ml of culture to the overall cfu per ml counts of the competent cells. Transformants, containing a stable pM272B plasmid were detected using PCR (section 2.3.13). Only transformants that showed presence of both KAN^r gene and the *sacB* gene by PCR were used for generating the *M. smegmatis* mutants.

2.3.15.1.2 Sucrose counter selection

M. smegmatis mc^2155 transposon mutants were generated using sucrose counter selection as follows. *M. smegmatis* mc^2155 transformants (section 2.3.15.1.1) were grown in 7H9 broth containing 25 μg/ml KAN overnight at 37^{0}C to an OD of 1.1 at 600 nm. The cells were repeatedly passed through a 26-gauge needle, before several dilutions of the cultures were plated on 7H11 agar plates containing 25 μg/ml KAN and 10% sucrose (negative selection). Transposon insertion mutants were obtained after 4-5 days incubation at 37^{0}C. Transposon mutants were checked for the loss of the *sacB* gene using PCR, and random insertion of the transposon into the mutants' genome was checked using DNA hybridization (section 2.3.14). The transposition frequency was estimated as a proportion of mutants generated to the number of cfu per ml of transformants' culture.

About 3000 of the *M. smegmatis* mc^2155 transposon mutants generated were picked into 96-well plates containing 150 μl 7H9 broth with 25 μg/ml KAN and incubated for 2 days at 37^{0}C. The appropriate volume of DMSO to make up 8% of the total culture volume in each well was then added and the plates were stored at -80^{0}C.

2.3.15.2 Mutants screening and isolation of biocides mutants

The process of screening and isolation of *M. smegmatis* mc^2155 biocides mutants is shown in Figure 2.7. Briefly, triclosan, cetylpyridium chloride, chlorhexidine diacetate, and *ortho*-phthalaldehyde mutants were isolated by replica plating from the 96-well plates onto first master 7H11 plates containing 25 μg/ml KAN and onto selective 7H11 plates with 25 μg/ml KAN and containing different screening concentrations of biocides. These concentrations were as follows: triclosan at 0.4, 0.6, 0.8, 1, and 1.2 μg/ml, *ortho*-phthalaldehyde at 1400, 1600, 1800, 2000, and 2200 μg/ml, cetylpyridium chloride at 0.5, 2, 4, 5, and 8 μg/ml, chlorhexidine diacetate at 0.1, 0.25, 0.5, 0.75, and 1 μg/ml. After 2 days incubation at 37^{0}C, those mutants which grew on the master 7H11/KAN plates but not onto the selective, biocides containing plates, were picked and stored in 7H9 broth containing 8% DMSO at -80^{0}C. Colonies which grew at higher biocide

concentrations were also isolated for probable resistance. These mutants were further characterised both in solid and liquid media, along with two random mutants which showed no changes in sensitivity to any of the biocides and which were used as controls.

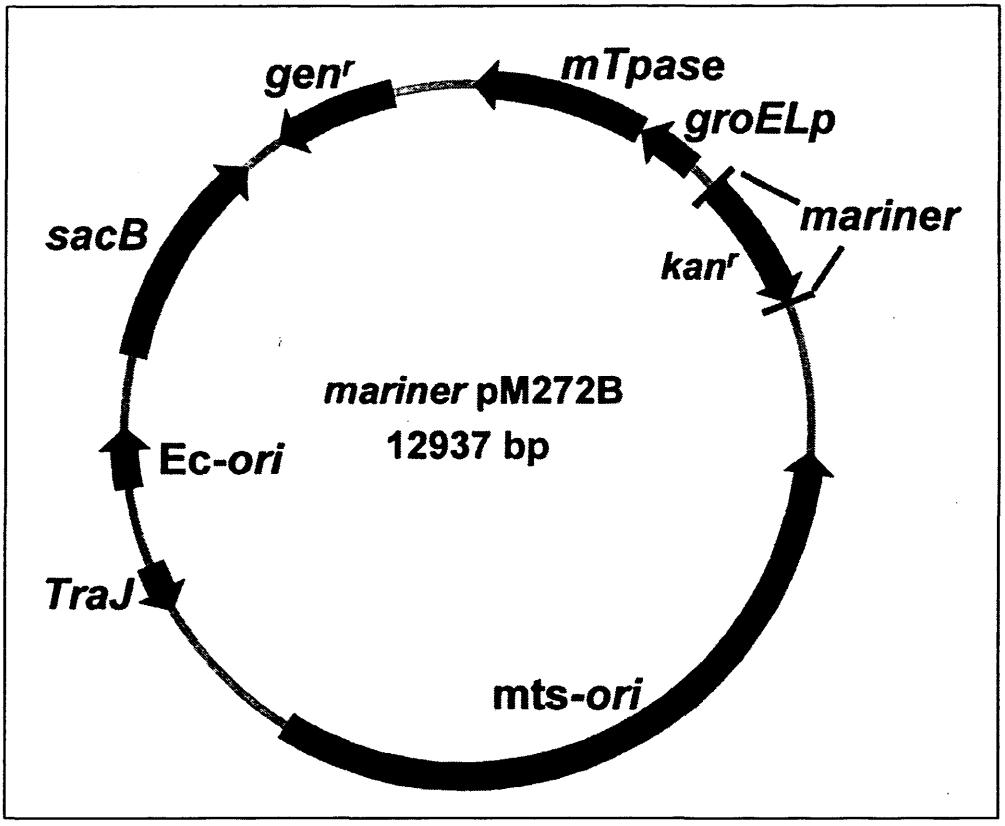


Figure 2.5. Map of the *mariner* transposon mutagenesis vector pM272B. Adapted from Gao *et al.* (324).

The vector was constructed by inserting a *mariner* transposon cassette into the plasmid backbone of pPR27 (812), a mycobacterial shuttle vector that contains the levansucrase (*sacB)* gene from *Bacillus subtilis* (1012), the GEN^r marker, an *E. coli* origin of replication (Ec-*ori*), and a thermosensitive *Mycobacterium ori* (mts-*ori*). The *mariner* transposon cassette contains the following elements: the 5' and 3' inverted repeats of *Mos1* surrounding the KAN^r cassette of Tn*903* (from *MosK* CD (1066)) and the *Mos1* transposase (*mTpase* from pET3a-Tpase (1066)), which was under the control of the mycobacterial *groEL* promoter (*groELp* from pSMT3 (325)).

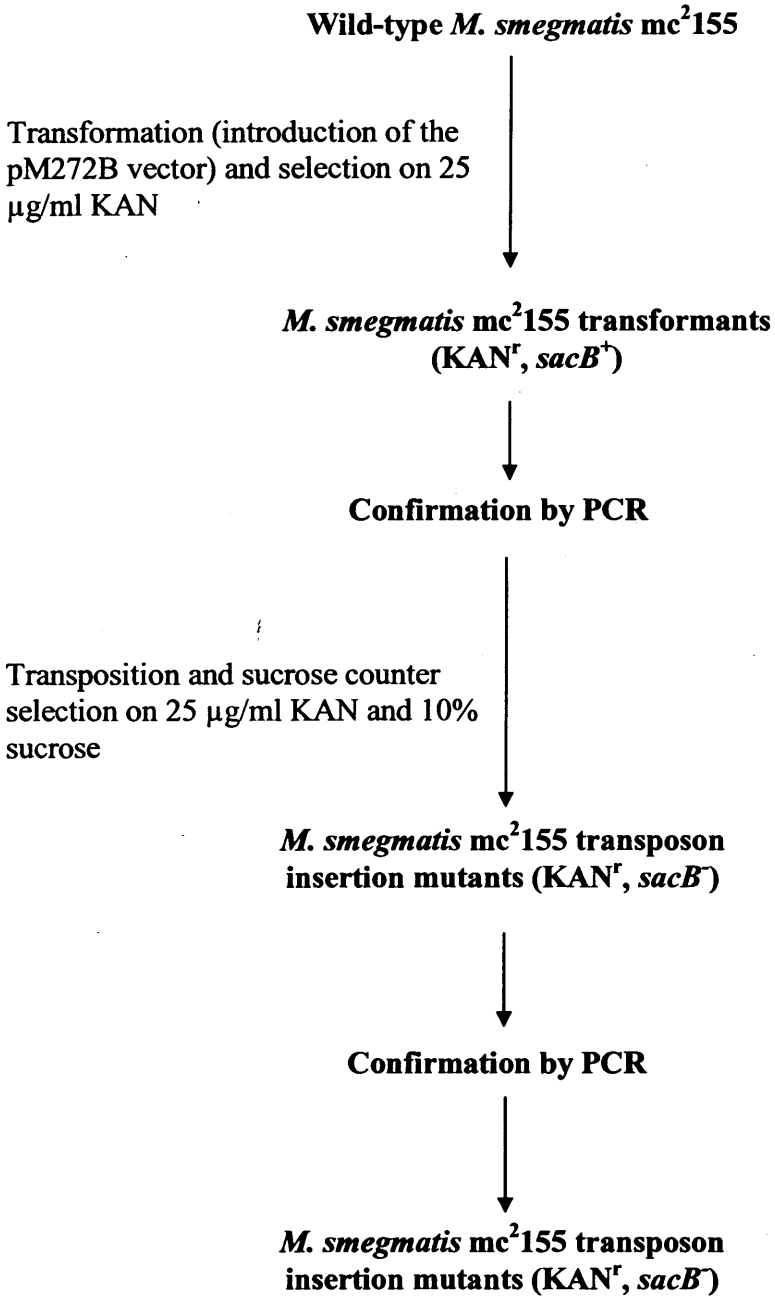


Figure 2.6. Generation of the *M. smegmatis* mc²155 transposon insertion mutants.
Transposon insertion mutants were generated using a two-step selection procedure, first on KAN, and second on KAN and sucrose. KAN^r, kanamycin resistant, *sacB*^-, *sacB* deficient.

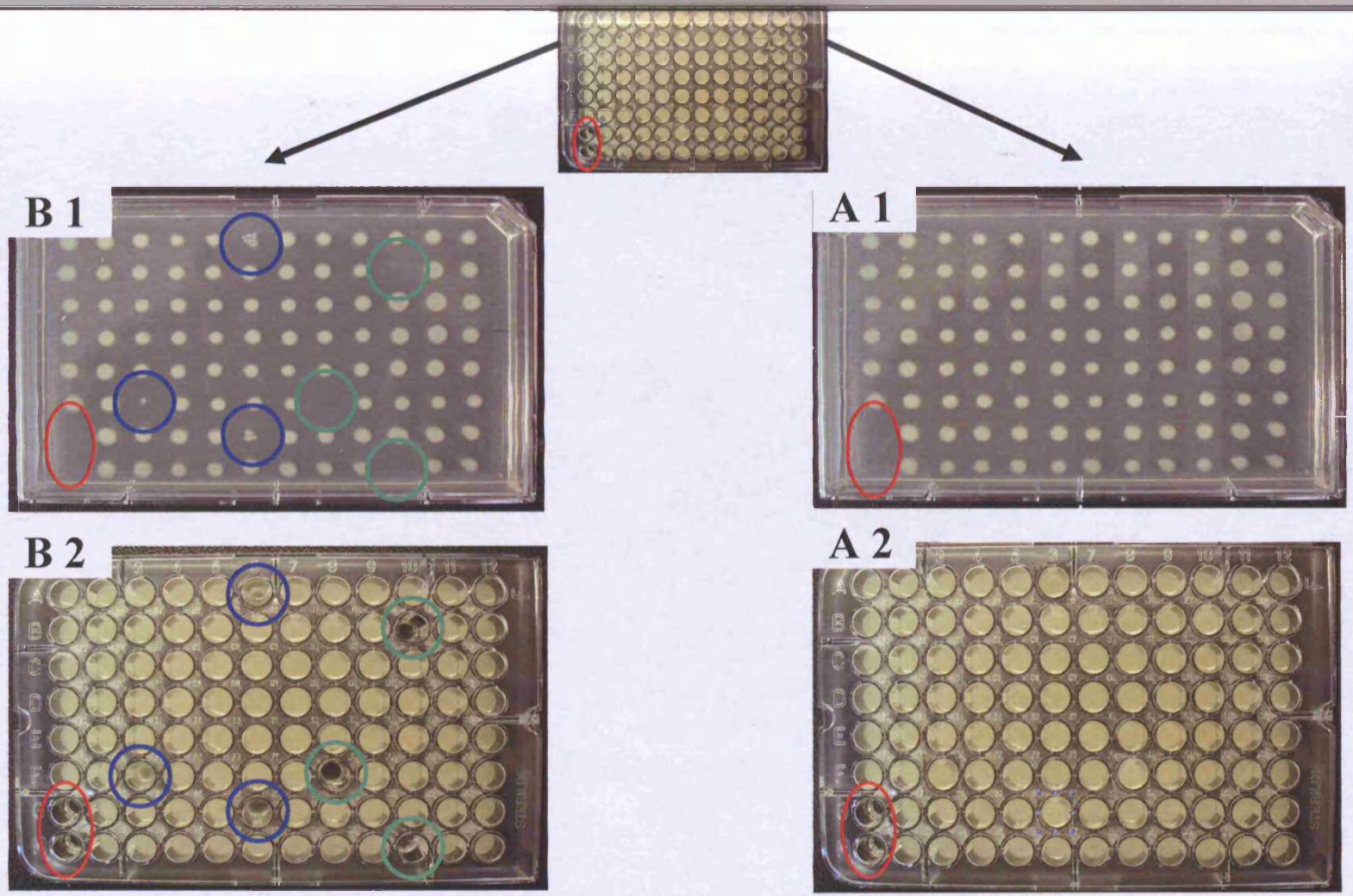


Figure 2.7. Mutants screening and isolation of biocides sensitive mutants.

Illustration of the general procedure used to isolate biocide susceptible mutants in this study. Biocide sensitive mutants (*S. marcescens* Db11 illustrated above) were isolated by replica plating of mutants from 96-well plates onto first a control agar plates (A1) containing the appropriate selection for mutants' growth and onto selective agar plates (B1) containing different screening concentrations of biocides. Mutants which grew on the control plates but showed either weak growth (blue circles) or no growth (green circles) on the selective biocides containing plates were picked for further characterisation. Mutants were screened a second time using liquid media (A2, B2) to confirm the agar screening results. Blanks (red circles) were included in all screening tests.

2.4 RESULTS

2.4.1 *M. smegmatis*

2.4.1.1 Growth characteristics

2.4.1.1.1 Colony morphology and growth on solid media

M. smegmatis mc^2155 grew well on both Middlebrook 7H11 agar, supplemented with 0.5% glycerol and 10% OADC, and TSA. Colonies started to appear after 24 h incubation at 37^0C, and reached optimal growth after 2 days incubation. Colonies appeared smooth and non-pigmented in the early stages of growth, however with prolonged incubation (2-7 days), the growth became thick, coarsely wrinkled, and colonies developed a yellow-orange pigmentation (Figure 2.8). Approximately 95% of the *M. smegmatis* sensu stricto isolates develop this characteristic pigmentation (108).

2.4.1.1.2 Growth in liquid media

Biocides activity on bacteria is affected by organic load, hence it was appropriate to determine the biocide MICs for *M. smegmatis* mc^2155 both in organic rich media (7H9 broth) and media with less organic matter. Therefore, growth of *M. smegmatis* mc^2155 in liquid media with different degrees of organic material was tested. These media were: TSB, Lemco broth, 7H9 broth, and Sauton medium. Growth was determined by following OD readings at 630 nm, generated by automated plate reader, over time (section 2.3.5).

Results showed that all media were able to sustain growth of *M. smegmatis* mc^2155. However, there were differences in the amount of growth reached in each medium after 2 days incubation at 37^0C. The organism grew best in 7H9 broth reaching an OD of over 1.6. Growth in the other media, as expected, was not optimal and culture ODs reached a value of just over 1 after 2 days. The results showed that good growth of *M. smegmatis* mc^2155 can be achieved in rich media such as 7H9 broth, although

other media such as TSB and Sauton can sustain adequate growth of the organism. Both 7H9 and TSB broths were used in further biocide testing experiments.

2.4.1.1.3 *M. smegmatis* mc^2155 growth curve

The different stages of growth of *M. smegmatis* mc^2155 and their length were determined to help with subsequent screening and tests. Growth curves of the organism were generated by reading ODs at 630 nm (section 2.3.5) of *M. smegmatis* mc^2155 cultures growing in 100 ml of 7H9 broth at 37^0C with gentle shaking. The curves were then used to determine the mean exponential growth rate and mean doubling time for the organism. The *M. smegmatis* mc^2155 cultures showed all the characteristic stages of bacterial growth (Figure 2.9). It had a short (1-2 h) "lag phase" as the medium was inoculated with a freshly grown *M. smegmatis* mc^2155 culture. The exponential "log phase" of growth extended over approximately 15 h with a mean growth rate value of $\mu = 0.21\ h^{-1}$, and mean doubling time of 3.3 h. The "stationary phase" started with a deceleration phase and was the longest phase of growth extending over 20 h.

2.4.1.1.4 Relationship between optical density and viable count

The relationship between the OD values at 630 nm of *M. smegmatis* mc^2155 cultures and their viable counts was also investigated (section 2.3.6) to determine the number of cfu present per ml of a standard inoculum used in further investigations in this study. The standard inoculum chosen was a culture with an OD (630 nm) of 0.5. The results (Figure 2.10) showed that the relationship between the OD values and the viable count was linear, and the relationship remained uniform up to an OD of over 1. *M. smegmatis* mc^2155 culture with an OD of 0.5 at 630 nm contained approximately 10^8 cfu/ml of bacteria.

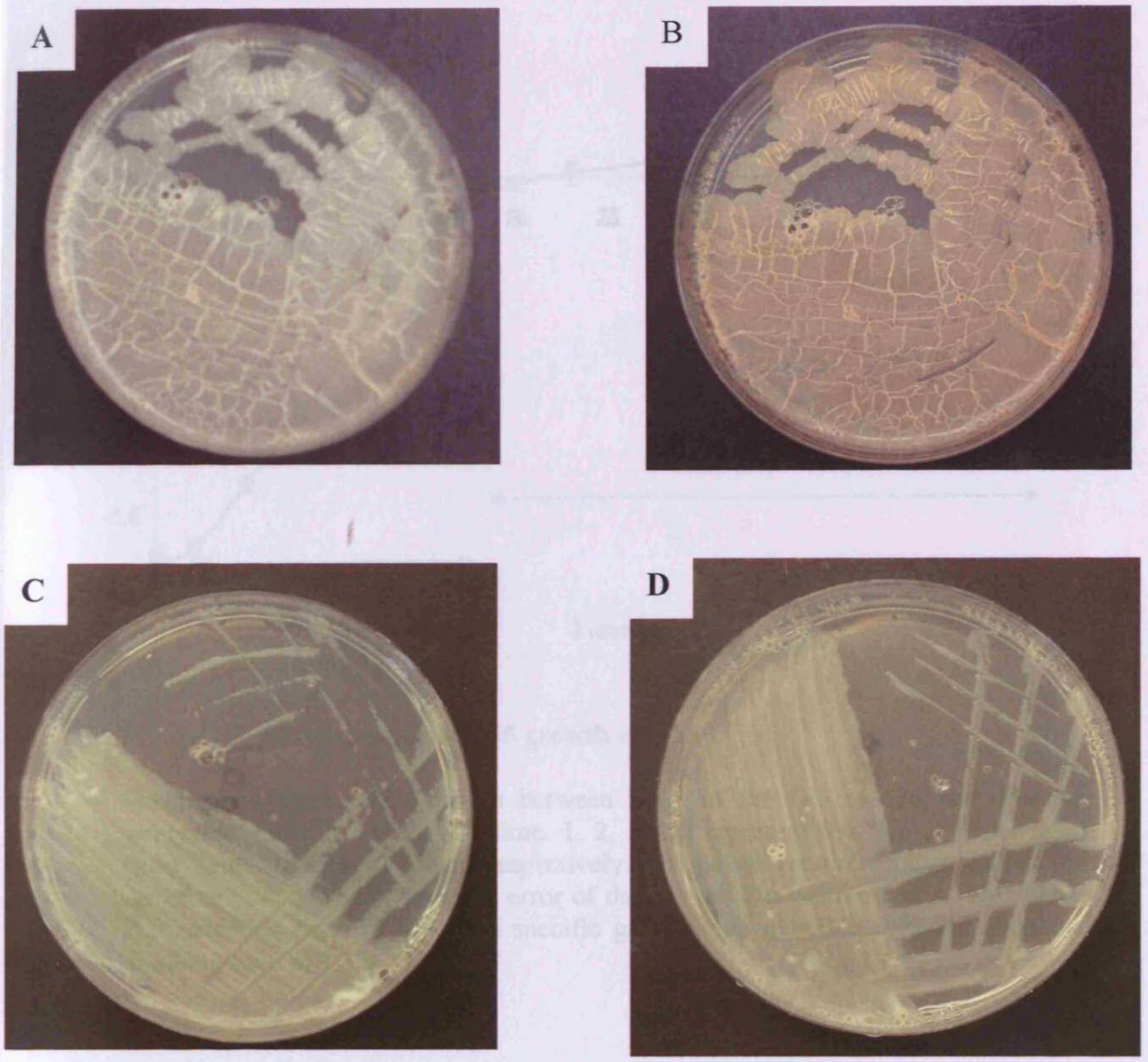


Figure 2.8. Colony morphology of the mycobacterial species studied.

A; *M. smegmatis* grown for 2 days at 37^0C, B; "A" after long incubation (3-7 days), C; *M. abscessus* grown at 37^0C for 3 days, D; *M. chelonae* grown at 30^0C for 3 days. *M. smegmatis* developed a late yellow pigmentation.

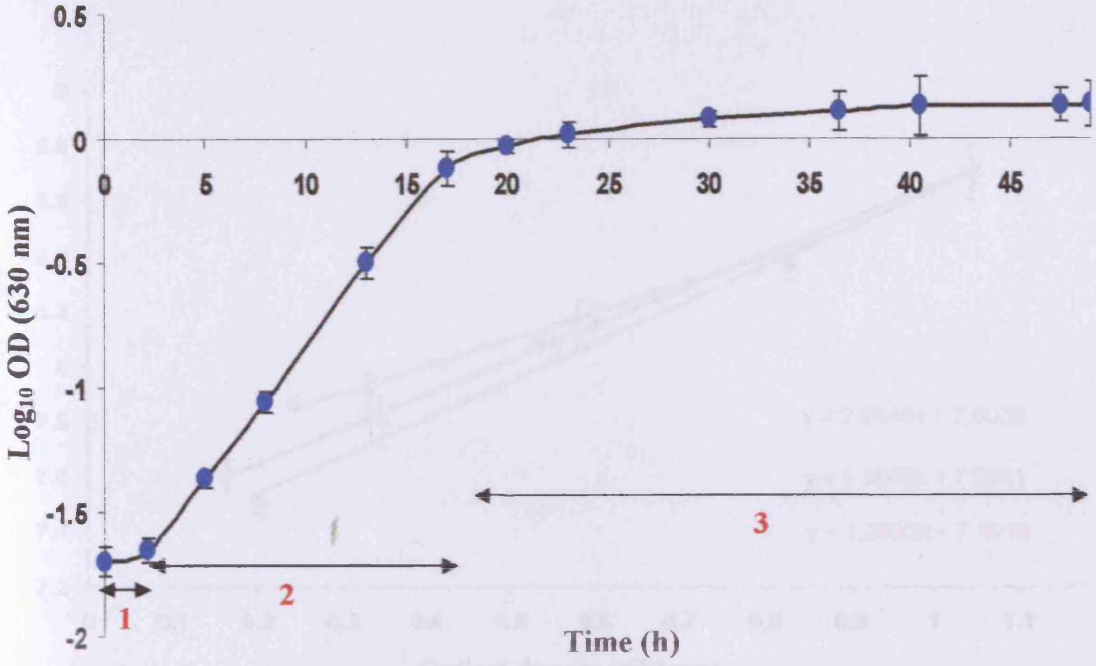


Figure 2.9. *M. smegmatis* mc^{2}155 growth curve.

Graph showing the relationship between $\log_{10}$ of the OD at 630 nm of a *M. smegmatis* mc^{2}155 culture and time. 1, 2, and 3 represent the "lag", "exponential (log)" and "stationary" phases respectively. The points represent mean values and the error bars represent standard error of the means. The culture had a "log" phase that extended over 15 h with a specific growth rate $\mu = 0.21\ h^{-1}$, and a culture doubling time of 3.3 h.

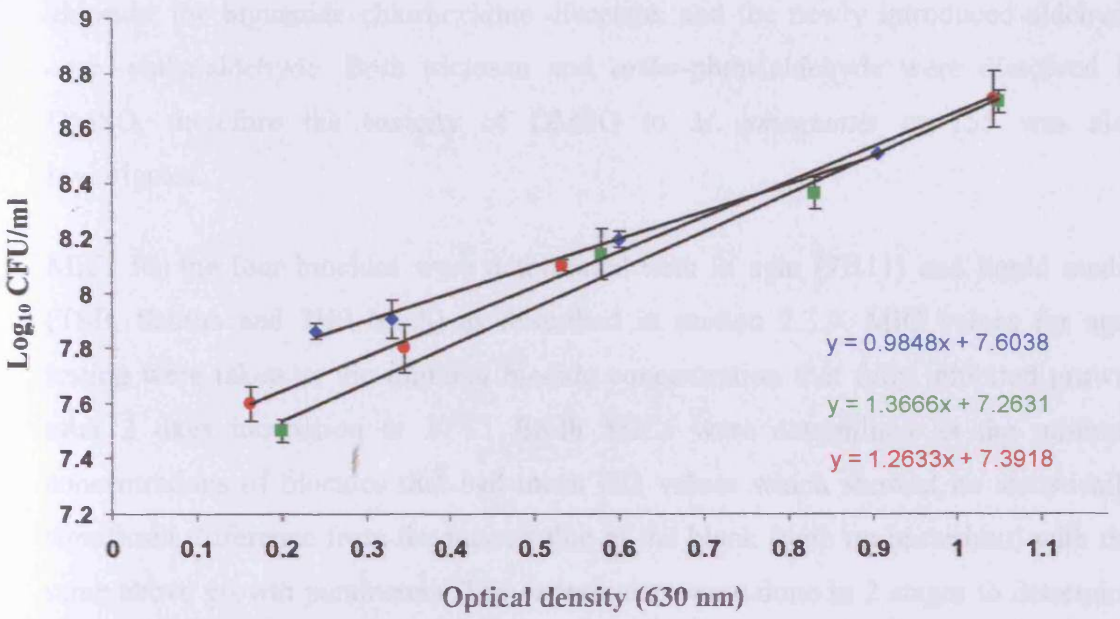


Figure 2.10. Relationship between OD and viable count for the mycobacterial species studied.

The lines are the best fit for the values, and their equations are shown on the graph. The points represent mean values and the error bars represent standard error of the means. Symbols: ● ; *M. chelonae*, ◆ ; *M. smegmatis*, ■ ; *M. abscessus*.

2.4.1.2 Biocide MICs for *M. smegmatis*

Susceptibility of *M. smegmatis* mc^2155 to four biocides was investigated by determining the MICs of these biocides for the bacterium (section 2.3.9). The four biocides used were the widely used bis-phenol triclosan, the QAC, cetylpyridinium chloride, the biguanide chlorhexidine diacetate, and the newly introduced aldehyde *ortho*-phthalaldehyde. Both triclosan and *ortho*-phthalaldehyde were dissolved in DMSO, therefore the toxicity of DMSO to *M. smegmatis* mc^2155 was also investigated.

MICs for the four biocides were determined both in agar (7H11) and liquid media (TSB, Sauton and 7H9 broth) as described in section 2.3.9. MIC values for agar testing were taken as the minimal biocide concentration that fully inhibited growth after 2 days incubation at 37^0C. Broth MICs were determined as the minimal concentrations of biocides that had mean OD values which showed no statistically significant difference from the mean value of the blank (with no bacterium) with the same above growth parameters. The experiments were done in 2 stages to determine accurate MIC concentrations of the biocides; initially MICs were evaluated over a wide range of biocide concentrations, once an approximate biocide MIC level had been determined, MICs were re-evaluated across a narrow concentration range.

The results (Table 2.10, Figure 2.11) showed that in liquid media, DMSO concentrations above 2% did have an effect on growth of the bacterium. The minimal DMSO concentrations that completely inhibited growth were 8% and 10% in TSB and 7H9 broth respectively. The maximum concentration of DMSO used in biocide MIC determination was 2%, hence DMSO did not have a significant effect on *M. smegmatis* mc^2155 growth in this study.

Liquid MIC values (Table 2.10, Figure 2.11) were higher in the rich 7H9 broth than those in media with less organic content (TSB, Sauton), as biocide action is slowed in the presence of organic matter. Chlorhexidine diacetate was the most effective biocide at inhibiting the growth of *M. smegmatis* mc^2155 in both solid and liquid media. It had MIC values of 3 μg/ml, 2.5 μg/ml, and 2 μg/ml in 7H9 broth, TSB, and Sauton medium respectively, and 0.75 μg/ml in 7H11 agar. Triclosan was the second most effective biocide at inhibiting *M. smegmatis* mc^2155 growth in solid agar, and had

MIC values of 1 μg/ml. Low concentrations of cetylpyridium chloride were also effective at inhibiting *M. smegmatis* mc^2155 growth. The agent had MIC values of 7.5 μg/ml, 3.5 μg/ml, 4.5 μg/ml and 5 μg/ml in 7H9 broth, TSB, Sauton medium and 7H11 agar respectively. The agar MIC for *ortho*-phthalaldehyde was much higher than that of the other three agents, with a value of 2000 μg/ml. Broth MIC of triclosan and *ortho*-phthalaldehyde were not determined because the interaction between *ortho*-phthalaldehyde and the bacterial suspensions resulted in change in the color of the medium (turning into a milky white color with increasing concentrations of triclosan, and a dark color, ranging from green to black, depending on concentration, with *ortho*-phthalaldehyde), hence making reading ODs impossible.

Table 2.10. Biocide MICs for *M. smegmatis* mc^{2}155.

Agents	MIC µg/ml (± SD)			
	Agar	Broth		
	7H11	7H9	TSB	Sauton
Chlorhexidine diacetate (CHX)	0.75 (0.3)	3 (1.4)	2 (0)	2.5 (2.1)
Cetylpyridinium chloride (CPC)	5 (0.7)	7.5 (3.5)	3.5 (2.1)	4.5 (2.1)
Triclosan (TRI)	1 (0)	Nd	Nd	Nd
Ortho-phthalaldehyde (OPA)	2000 (700)	Nd	Nd	Nd
Dimethyl sulfoxide (DMSO)*	Nd	10 (2.8)	8 (2.8)	Nd

* [DMSO] expressed in % (v/v)
Nd; not determined.

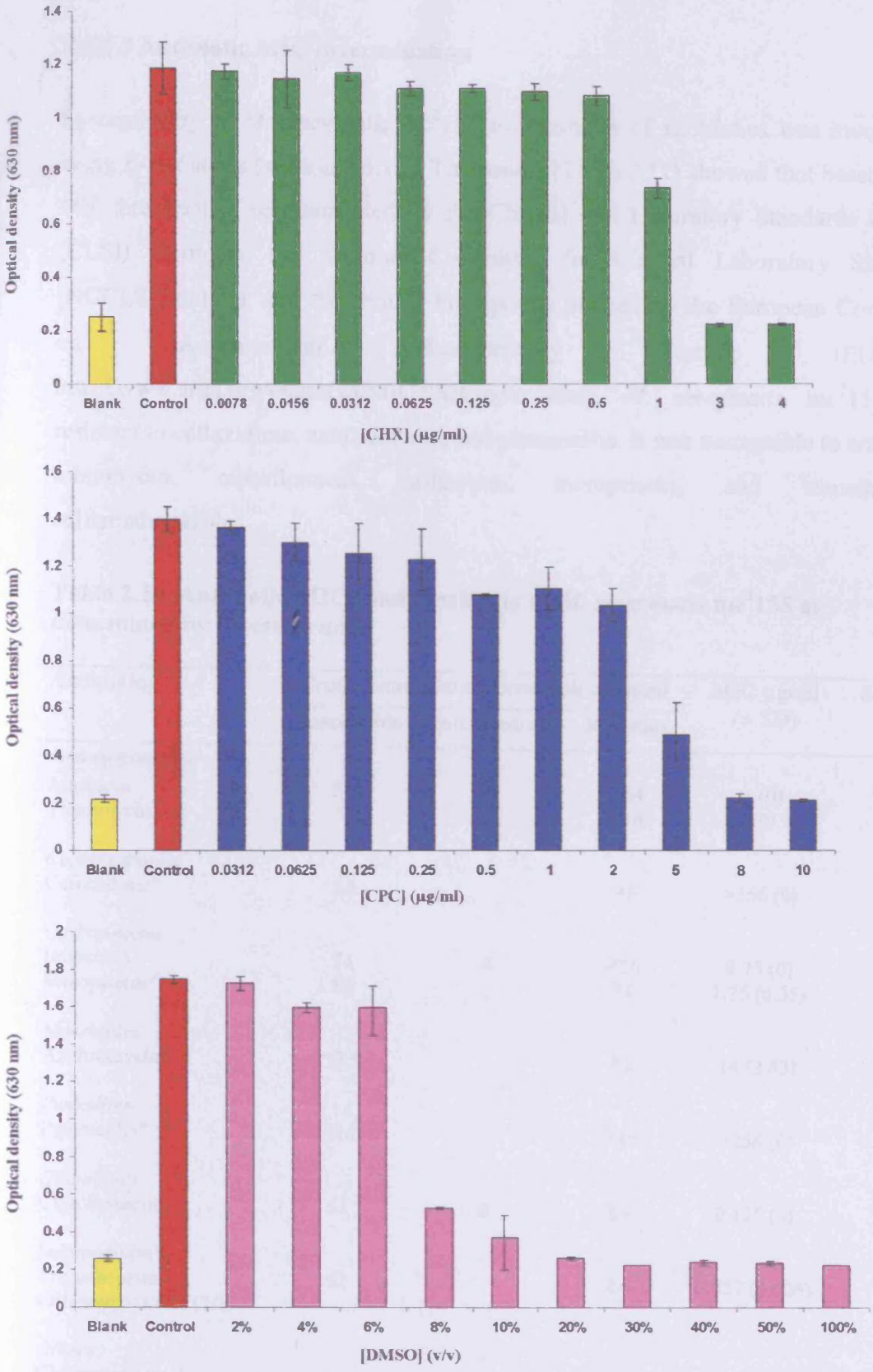


Figure 2.11. Broth MICs of cetylpyridinium chloride, chlorhexidine diacetate, and DMSO for *M. smegmatis* mc^{2}155.

MICs were determined in 7H9 broth supplemented with the appropriate concentration of agents. Optical densities were read after 2 day incubation at 37^{0}C. CHX; chlorhexidine diacetate, CPC; cetylpyridinium chloride, DMSO; dimethyl sulfoxide.

2.4.1.3 Antibiotic MIC determination

Susceptibility of *M. smegmatis* mc^2155 to a number of antibiotics was investigated using E-test strips (section 2.3.10). The results (Table 2.11) showed that based on the MIC breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) (formally the National Committee for Clinical Laboratory Standards [NCCLS]) (1160), and the clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST: http://www.srga.org/eucastwt/MICTAB/index.html), *M. smegmatis* mc^2155 was resistant to ceftazidime, azithromycin, and piperacillin. It was susceptible to amikacin, tobramycin, ciprofloxacin, imipenem, meropenem, and trimethoprim-sulfamethoxazole.

Table 2.11. Antibiotic MICs and sensitivity of *M. smegmatis* mc^2155 as determined by E-test strips.

Antibiotic	Broth microdilution breakpoints µg/ml			MIC µg/ml (± SD)	Sensitivity
	Susceptible	Intermediate	Resistant		
Aminoglycosides					
Amikacin	≤16	32	≥64	1 (0)	S
Tobramycin	≤4	8	≥16	2.5 (0.7)	S
Cephalosporins					
Ceftazidime*	≤4		≥8	>256 (0)	R
Carbapenems					
Imipenem	≤4	8	≥16	0.75 (0)	S
Meropenem*	≤2		≥8	1.75 (0.35)	S
Macrolides					
Azithromycin*	<0.5		>2	14 (2.83)	R
Penicillins					
Piperacillin*	<16		>16	>256 (0)	R
Quinolones					
Ciprofloxacin	≤1	2	≥4	0.125 (0)	S
Sulfonamides					
Trimethoprim-sulfamethoxazole (1/19)	≤2		≥4	0.027 (0.006)	S
Others					
Chloramphenicol	-		-	16 (8)	Nd

Drugs and breakpoints recommended by NCCLS document M24-T2 (1160). * Based on clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) http://www.srga.org/eucastwt/MICTAB/index.html. Nd; not determined.

2.4.1.4 Mutant generation

M. smegmatis mc^2155 was transformed with the *mariner*-based transposon mutagenesis vector pM272B (Figure 2.5). The vector was first used by Gao *et al.* (324) to successfully generate *M. marinum* transposon mutants. It was constructed by inserting a *mariner* transposon cassette into the plasmid backbone of pPR27 (812). The *mariner* family of transposable elements is named for the original element discovered in *Drosophila mauritiana* (409). They are transposons of the short inverted terminal repeat class and very useful molecular tools.

Plasmid pM272B was extracted from the host *E. coli* JM109 (section 2.3.11.2), and checked on an agarose gel (section 2.3.12), before being used in transformation experiment (section 2.3.15.1). As *M. smegmatis* mc^2155 was sensitive to 25 μg/ml kanamycin, *M. smegmatis* mc^2155 transformants containing the vector (hence the KAN resistance (KAN^r) gene) were selected on agar contaning the above concentration of kanamycin. Transposon mutants were isolated using sucrose counter-selection (section 2.3.15.1.2). Frequency of spontaneous resistance to KAN in *M. smegmatis* mc^2155 and the efficiencies of transposition (transposon mutation) and pM272B transformation in *M. smegmatis* mc^2155 were determined. These were expressed as frequency of competent mc^2155 cells used for transformation to transformants collected, to determine transposition efficiency, and frequency of transformants to mutants generated, to determine transposition (mutation) frequency.

The spontaneous KAN resistance frequency of *M. smegmatis* mc^2155 was 10^{-7}, in the same order as that reported by Snapper *et al.* (993), who calculated the spontaneous mutation frequency of mc^2155 and its parental strain mc^26 for this agent to be 10^{-7}-10^{-8}. Although the pM272B transformation frequency was low (in the order of 10^{-5}), only one transformant containing the pM272B was needed for subsequent successful counterselection. The frequency of transposition was much higher (10^{-2}) and 3000 *M. smegmatis* mc^2155 transposon mutants were isolated.

Since the vector contained the *mariner* transposon with a KAN^r gene for positive selection and a *sacB* gene for counterselection, the mc^2155 transformants should have contained both the KAN^r and *sacB* genes. In contrast, the mutants which should have

had chromosomal insertion(s) of *mariner*, with loss of the delivery vector, would therefore have just contained the KAN^r gene with loss of the *sacB* gene. This was tested by examining the presence of both the KAN^r and the *sacB* genes in a number of randomly picked mc^2155 transformants and mutants using PCR (section 2.3.13). Results of the PCR amplification (Figure 2.12) demonstrated that mutants possessed the correct genotype. For the KAN^r gene amplification, products of the expected size ($\approx$ 620 bp) were obtained with DNA from the pM272B control and all transformants and mutants tested (Figure 2.12), which indicates that they all contained the KAN^r gene. In the *sacB* gene amplification, PCR products of expected size ($\approx$ 1422 bp) were obtained with DNA from the pM272B vector and the transformants. No PCR products were obtained with DNA from the mutants (Figure 2.12). This was in accordance with the fact that none of the mutants should contain a copy of the *sacB* gene due to loss of the delivery vector after the transposition event. Results of the PCR amplification suggested successful transformation of the *M. smegmatis* mc^2155 strain with the pM272B vector and integration of the transposon into the chromosome.

DNA hybridization was used to check for single random insertion of the *mariner* transposon into the *M. smegmatis* genome. Southern blots of *Pst*I, *Hind*III and *Sal*I (none of these enzymes cuts within the probe sequence) digested genomic DNA from randomly selected mc^2155 mutants were probed with the KAN^r gene sequence (Figure 2.13). Both positive and negative controls were included and they were *M. smegmatis* mc^2155 genomic DNA, which does not contain the KAN^r gene, as a negative control, and vector DNA as a positive control. There was no hybridization band seen in any Southern blot using *M. smegmatis* mc^2155 genomic DNA digest, and a single band was seen in all blots when vector DNA digests were used. In most mutants, Southern blot yielded a single hybridization fragment, suggesting a single transposition event for each mutant. In some mutants (e.g. mutant 5, Figure 2.13) more than one band was seen. This could be due to a number of reasons including genomic rearrangement in the mutants, mutants colonies were not pure, or simple multiple transposition of the *mariner* system. The latter was reported by Gao *et al.*(324) in 4% of *M. marinum* mutants generated using the same transposon system. The Southern blot results also showed that many of the hybridization fragments had a unique size, indicating that transposon had inserted at different sites in the chromosome. Taken together, these results proved that the mutant generation protocol (Figure 2.6) used lead to integration

of the mariner transposon into the chromosome of *M. smegmatis* mc^2155 mainly at random sites and usually as a single copy per genome.

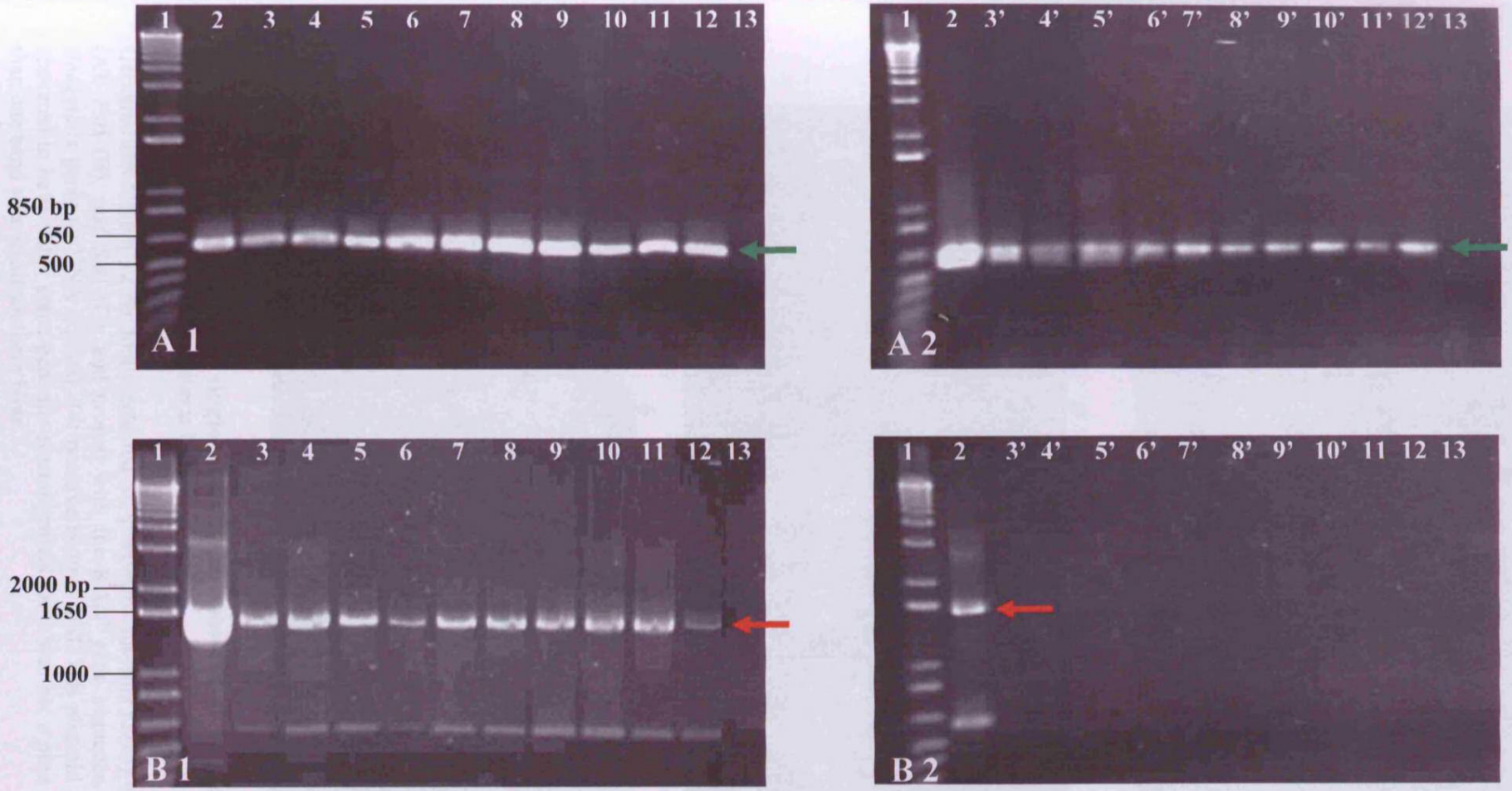


Figure 2.12. Confirmation of transformation and *mariner* element transposition into *M. smegmatis* mc^{2}155 by PCR.

Transformation of *M. smegmatis* with pM272B vector and subsequent insertion of the *mariner* transposon into the genome was tested by PCR amplification of two genes carried on the pM272B plasmid, the *sacB* gene (B1-2) and the KAN^r gene (A1-2) carried on the *mariner* part of the vector. Transformants containing the pM272B plasmid should prove positive for both genes, whereas mutants which lost the vector sequence after transposition of the *mariner* element, should be positive for the KAN^r gene (green arrows, ≈ 620 bp) and negative for the *sacB* gene (red arrows, ≈ 1422 bp). 1; 1-kb+ DNA ladder size marker, 2; pM272B DNA (positive control), 3-12; 10 randomly selected transformants, 3'-12'; 10 random mutants, 13; *M. smegmatis* mc^{2}155 DNA (negative control).

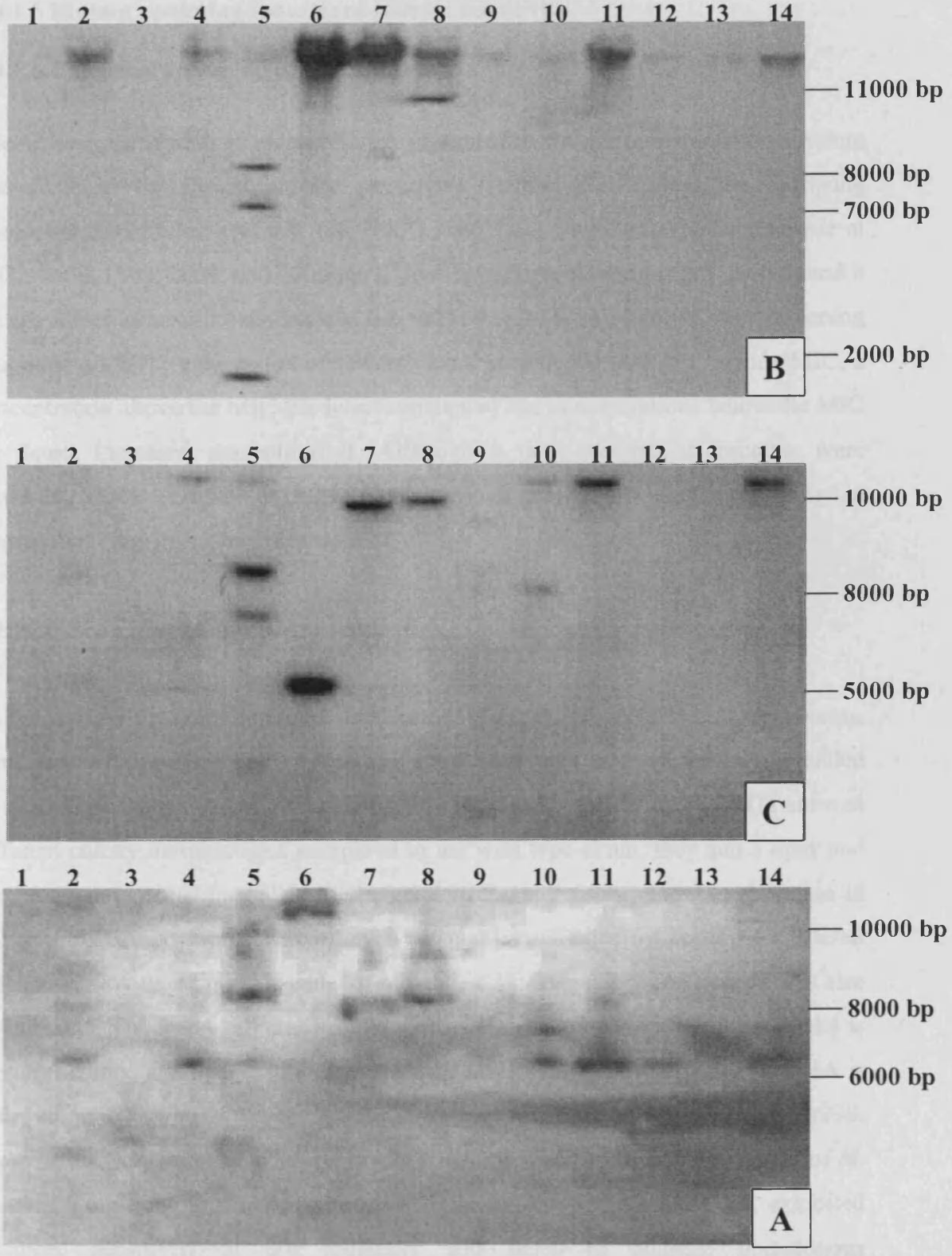


Figure 2.13. Confirmation of single random transposition of the *mariner* transposon in *M. smegmatis* mc^{2}155 by southern blot.

Genomic DNA from 12 randomly selected *M. smegmatis* mutants (2-13) was digested with *Hind*III (A), *Pst*I (B), and *Sal*I (C), and probed with the KAN^r gene sequence. 1; negative control (*M. smegmatis* genomic DNA digests), 14; positive control (pM272B plasmid DNA digests). Mutant 5 appeared to have either some genomic rearrangement, or it was not a pure colony, resulting in more than one band seen in all southern blots.

2.4.1.5 Mutant screening for altered biocide sensitivity

2.4.1.5.1 General screening of the mutants

The *M. smegmatis* mc^2155 mutant library generated by the *mariner*-transposon system was screened for altered biocide sensitivity (section 2.3.15.2) at the following concentrations: triclosan at 0.4, 0.6, 0.8, 1, and 1.2 μg/ml, *ortho*-phthalaldehyde at 1400, 1600, 1800, 2000, and 2200 μg/ml, cetylpyridium chloride at 0.5, 2, 4, 5, and 8 μg/ml, and chlorhexidine diacetate at 0.1, 0.25, 0.5, 0.75, and 1 μg/ml. The screening was done on 7H11 agar and at concentrations that included both the biocide MIC, a concentration above the MIC (to detect resistance) and concentrations below the MIC (to detect increased susceptibility). Although a large number of mutants were screened (3000), we failed to isolate mutants which showed any significant change in sensitivity to any of the biocides tested.

2.4.1.5.2 Screening of specific mutants

As the general screening failed to isolate any *M. smegmatis* mc^2155 biocide mutants, 6 mutants with possible cell surface alteration have been selected for more detailed screening. These mutants (10-A1, 2-B8, 15-E3, 3-A10, 2-A5, and 14-H1) showed different colony morphologies compared to the wild type strain, they had a drier and waxier appearance compared to the parental strain, suggesting possible alteration in cell surface composition. Hence they were good candidates for testing for altered biocide sensitivity. Along this panel of mutants, a *M. smegmatis recA* mutant was also investigated. The RecA protein lies in the heart of homologous recombination, and is a central component of the SOS response, and DNA repair (557, 1100). The RecA is both ubiquitous and well conserved among a range of prokaryotes. In 1998, Papavinasasundaram *et al.* (789) reported the isolation of a *recA* deletion mutant of *M. smegmatis* by homologous recombination. The mutant designated HS42, exhibited enhanced sensitivity to UV radiation, and failed to undergo homologous recombination. The mutant was successfully complemented with the *recA* gene from both *M. smegmatis* and *M. tuberculosis.* Due to the importance of the *recA* gene, it was interesting to investigate if deletion of this gene would have an effect on biocide sensitivity in *M. smegmatis.*

The altered morphology mutants along with the *recA* mutant were screened at more stringent concentrations in an attempt to detect any level of altered sensitivity for two of the biocides known to act on cell membrane, chlorhexidine diacetate and cetylpyridinium chloride. These concentrations were as follows: for chlorhexidine diacetate, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.2, and 1.4 μg/ml. For cetylpyridinium chloride, 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μg/ml. The result of the screening however did not show any alterations in sensitivity to these two biocides in any of the mutants tested. We concluded that a much larger mutant bank was probably needed to isolate the desired mutants.

2.4.2 *M. chelonae* and *M. abscessus*

M. chelonae and *M. abscessus*, two of the most antimicrobial resistant species of RGM, were used in this study, to determine their biocide and antibiotic susceptibility, and to compare their sensitivity with that of the *M. smegmatis* mc^2155. The two strains chosen were the type strains *M. chelonae* NCTC 946 and *M. abscessus* ATCC 19977.

2.4.2.1 Colony morphology and growth on solid media

Both strains of *M. chelonae* and *M. abscessus* grew well on Middlebrook 7H11 agar, supplemented with 0.5% glycerol and 10% OADC. The two strains were cultivated at different temperatures to achieve optimal growth, 37^0C for *M. abscessus* and 30^0C for *M. chelonae*. Slower growth, especially with *M. chelonae*, was observed on the less rich TSA. When cultured on the same agar plates, *M. smegmatis* mc^2155 grew well, whereas growth of both *M. chelonae* and *M. abscessus* was affected (Figure 2.14). In the presense of *M. smegmatis* mc^2155 both *M. chelonae* and *M. abscessus* showed slower and limited growth compared with their growth under the same conditions in the absence of *M. smegmatis* mc^2155 (Figure 2.14). This suggests that when present in the same environment with limited resources, competition for nutrients favours the faster growing *M. smegmatis* mc^2155 over *M. chelonae* or *M. abscessus*. Other possible explanation for the above observation is that *M. smegmatis* mc^2155 may be producing some inhibitory compound(s) that limits or inhibits the growth of other organism including *M. chelonae* and *M. abscessus*. This can only be speculated about

and further investigations are needed to prove or disprove this speculation. On agar, colonies of *M. chelonae* and *M. abscessus* started to appear after 2 days incubation, and reached optimal growth after 4 days. Colonies appeared smooth and did not develop pigmentation even after 7 days incubation (Figure 2.8).

2.4.2.2 *M. chelonae,* and *M. abscessus* growth curves and relationship between OD and viable count

The different stages of growth and their length were determined for both *M. chelonae* and *M. abscessus*. Growth curves of the organisms were generated from cultures growing in 7H9 broth and incubated at the appropriate temperature as described for *M. smegmatis* (section 2.4.1.1.3). The curves were then used to determine the mean exponential growth rates and mean doubling times for the organisms. *M. chelonae* and *M. abscessus* cultures both showed all the characteristic stages of bacterial growth (Figure 2.15). Both had a relatively short "lag phases" as the medium was inoculated with freshly grown cultures. However, there were differences in the length of the "lag phase" for the two organisms. Compared to *M. smegmatis*, the "lag phase" for *M. chelonae* was longer and extended over (5-6 h). *M. abscesus* had a "lag phase" length intermediate between that of *M. smegmatis* and *M. chelonae* (2-2.5 h).

The exponential "log phase" of growth of both organisms were relatively longer that that of *M. smegmatis*, extending over approximately 17 h and 22 h for *M. abscessus* and *M. chelonae* respectively. *M. chelonae* and *M. abscessus* grew slower than *M. smegmatis*, with *M. chelonae* being the slowest. Exponential growth rate and doubling time values were $\mu = 0.17\ h^{-1}$ and $g = 4.0$ h for *M. abscessus*, and $\mu = 0.15\ h^{-1}$ and $g = 4.6$ h for *M. chelonae*. The "stationary phase" was observed for both organisms after a deceleration period and started after 20 h and 30 h incubation for *M. abscessus* and *M. chelonae* respectively. Relationship between the OD readings of *M. chelonae* and *M. abscessus* cultures and their viable counts was also determined (Figure 2.10). Results showed that cultures with an OD value of 0.5 contained approximately 10^8 cfu/ml for both *M. chelonae* and *M. abscessus*.

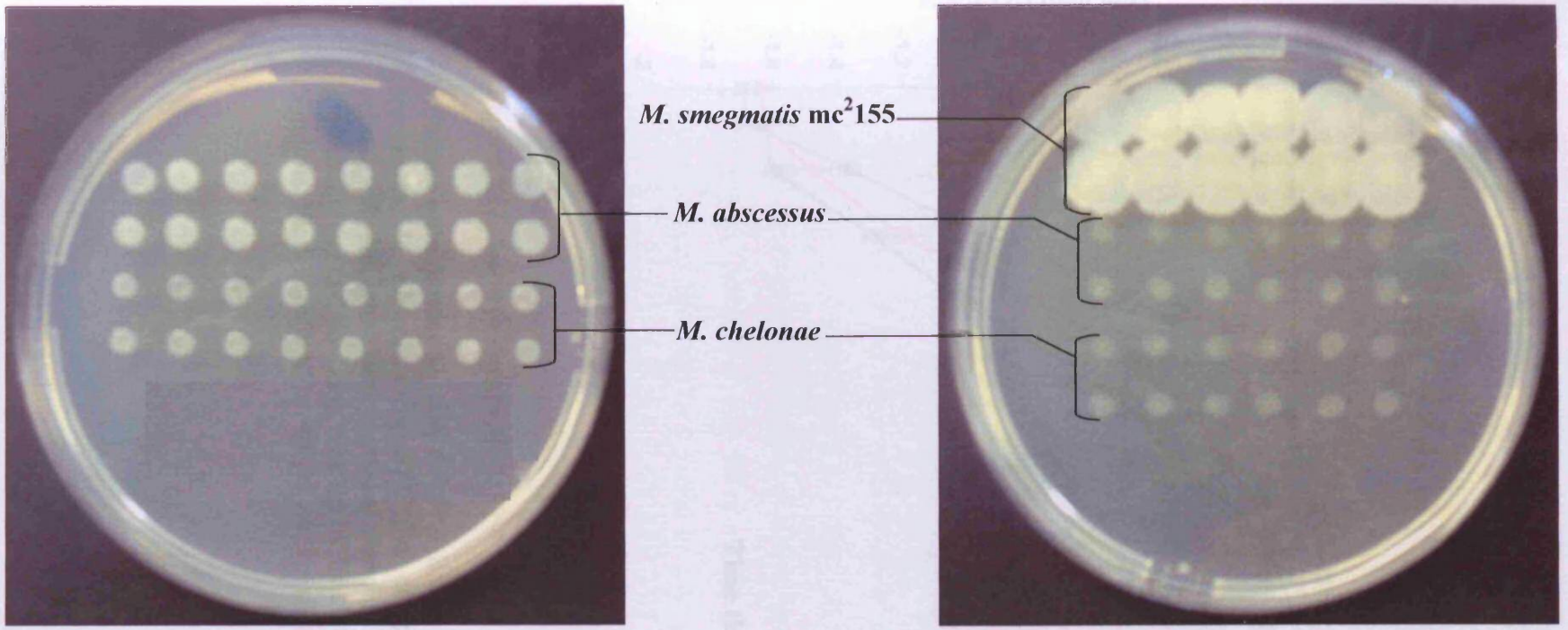


Figure 2.14. Effect of *M. smegmatis* growth on that of *M. chelonae* and *M. abscessus.*

Growth was tested on 7H11 agar and at 37^0C for 3 days. When *M. smegmatis* was present, both *M. abscessus* and *M. chelonae* showed slow and limited growth. When *M. smegmatis* was absent, *M. abcessus* and *M. chelonae* gew well.

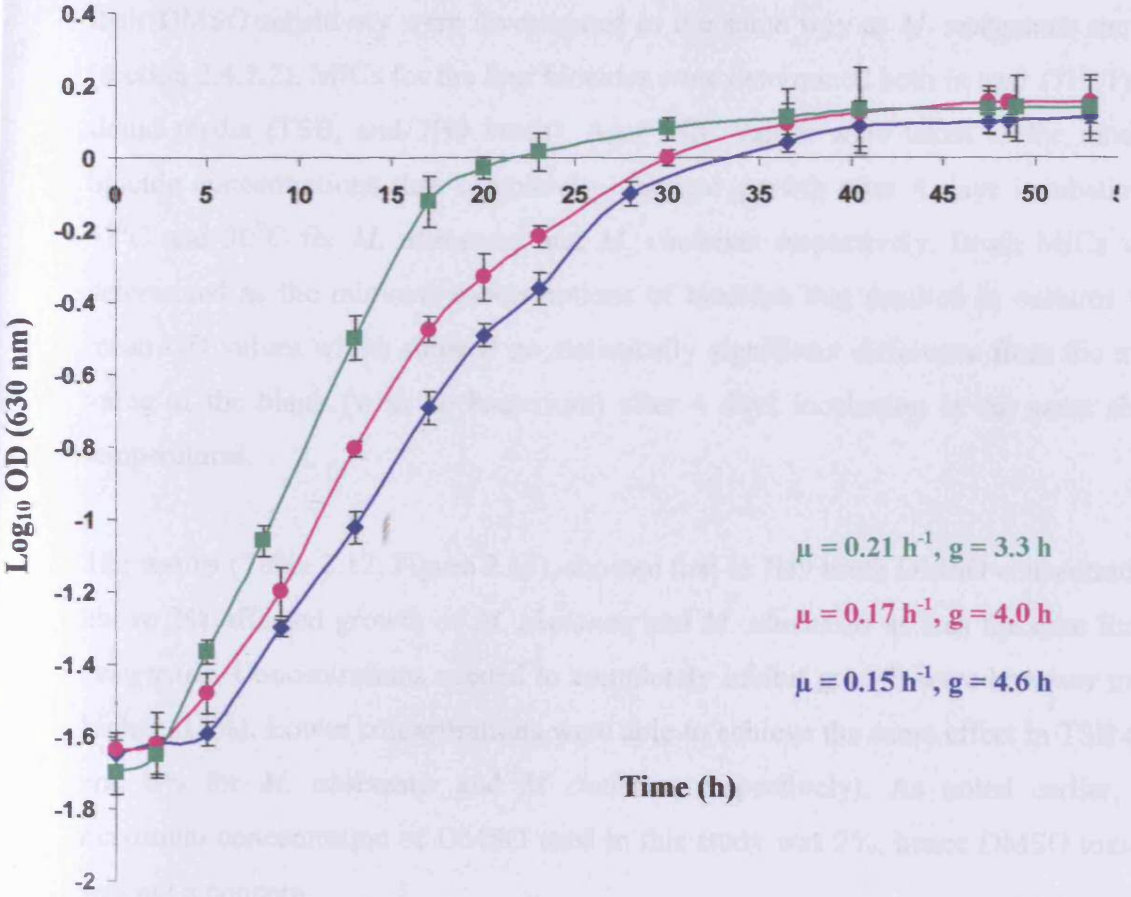


Figure 2.15. Growth curves of mycobacterial species studied.

Cultures were grown in 100 ml 7H9 broth with gentle shaking at 37^{0}C (*M. smegmatis*, *M. abscessus*) or 30^{0}C (*M. chelonae*). The points represent mean values and the error bars represent standard error of the means. Exponential growth rate (μ) and doubling time (g) of the cultures are shown on the graph. Symbols: ■; *M. smegmatis*, ◆; *M. chelonae*, ●; *M. abscessus*.

2.4.2.3 Biocide MICs for *M. chelonae* and *M. abscessus*

Susceptibility of *M. chelonae* and *M. abscessus* strains to four biocides (triclosan, cetylpyridinium chloride, chlorhexidine diacetate, and *ortho*-phthalaldehyde), and their DMSO sensitivity were investigated in the same way as *M. smegmatis* mc^2155 (section 2.4.1.2). MICs for the four biocides were determined both in agar (7H11) and liquid media (TSB, and 7H9 broth). Agar MIC values were taken as the minimal biocide concentrations that completely inhibited growth after 4 days incubation at 37^0C and 30^0C for *M. abscessus* and *M. chelonae* respectively. Broth MICs were determined as the minimal concentrations of biocides that resulted in cultures with mean OD values which showed no statistically significant difference from the mean value of the blank (with no bacterium) after 4 days incubation at the same above temperatures.

The results (Table 2.12, Figure 2.16), showed that in 7H9 broth DMSO concentrations above 2% affected growth of *M. chelonae* and *M. abscessus* as was the case for *M. smegmatis*. Concentrations needed to completely inhibit growth were however much higher (15%). Lower concentrations were able to achieve the same effect in TSB (8% and 6% for *M. abscessus* and *M chelonae* respectively). As noted earlier, the maximum concentration of DMSO used in this study was 2%, hence DMSO toxicity was not a concern.

MIC values for chlorhexidine diacetate in the two media used (7H9 broth and TSB) were comparable in all mycobacterial species, even though they contained different concentrations of organic matter. This was not the case with cetylpyridinium chloride, where the MIC values in 7H9 broth were much higher than those in TSB, especially for *M. abscessus* and *M. chelonae*. Another observation from the results, was that relatively low concentrations of triclosan, cetylpyridinium chloride and chlorhexidine diacetate were needed to inhibit growth in all mycobacterial species in both solid and liquid media (triclosan was not tested in broth). This was not the case with *ortho*-phthalaldehyde, where concentrations of 2000 µg/ml or above were needed to achieve the same effect. Chlorhexidine diacetate was the most mycobacteriostatic agent in both solid and liquid media for all the mycobacterial strains, followed closely by triclosan and cetylpyridinium chloride. The results also showed that there are

differences in biocide susceptibility for mycobacterial strains. For example, in the case of chlorhexidine diacetate, *M. abscessus* was much more resistant to this agent that the other two species, with the *M. smegmatis* mc^2155 being most sensitive. *M. smegmatis* strain was more sensitive to all agents tested than were the other two species which shower varying degrees of resistance in comparison.

Table 2.12. Biocide MICs and DMSO toxicity for mycobacterial species studied.

Agent	*M. smegmatis* mc^{2}155			*M. abscessus* ATCC 19977			*M. chelonae* NCTC 946		
	Agar MIC*	**Broth MIC***		**Agar MIC***	**Broth MIC***		**Agar MIC***	**Broth MIC***	
	7H11	7H9	TSB	7H11	7H9	TSB	7H11	7H9	TSB
CHX	0.75 (0.3)	3 (1.4)	2 (0)	8.75 (8.8)	7.5 (3.5)	7.5 (3.5)	3.75 (1.7)	2 (0)	2 (1.4)
CPC	5 (0.7)	7.5 (3.5)	3.5 (2.1)	25 (7)	70 (14)	10 (0)	30 (14)	45 (7)	5 (2.8)
OPA	2000 (700)	Nd	Nd	2250 (354)	Nd	Nd	2250 (354)	Nd	Nd
TRI	1 (0)	Nd	Nd	25 (7)	Nd	Nd	22.5 (3.5)	Nd	Nd
DMSO#	Nd	10 (2.8)	8 (2.8)	Nd	15 (7)	8 (2.8)	Nd	15 (7)	6 (0)

TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate, DMSO; dimethyl sulfoxide, Nd; not determined
* MIC values expressed in μg/ml (± standard deviation)
# [DMSO] expressed in % (v/v)

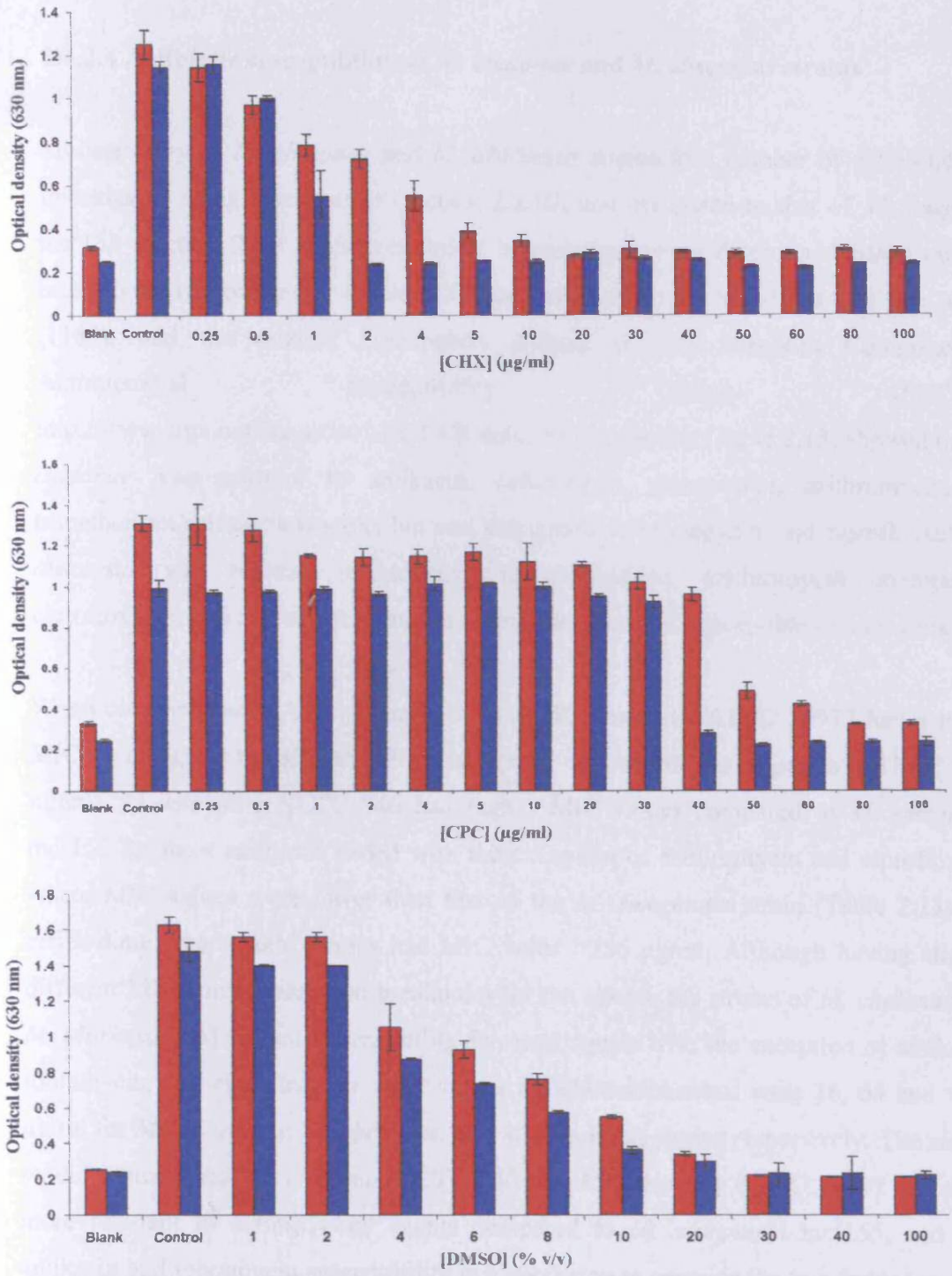


Figure 2.16. Broth MICs of cetylpyridinium chloride, chlorhexidine diacetate, and DMSO for *M. chelonae and M. abscessus* strains.

MICs were determined in 7H9 broth supplemented with the appropriate concentration of agents. Optical densities were read after 4 day incubation at 37^{0}C and 30^{0}C for *M. abscessus* and *M. chelonae* respectively. Error bars represent standard error of the means. CHX; chlorhexidine diacetate, CPC; cetylpyridinium chloride, DMSO; dimethyl sulfoxide. ■; *M. abscessus*, ■; *M. chelonae*.

2.4.2.4 Antibiotic susceptibility of *M. chelonae* and *M. abscessus* strains

Susceptibility of *M. chelonae* and *M. abscessus* strains to a number of antibiotics was investigated using E-test strips (section 2.3.10) and compared to that of *M. smegmatis* mc^2155 (section 2.4.1.3). Susceptibility or resistance were determined based on MIC breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) (1160), and the clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST: http://www.srga.org/eucastwt/MICTAB/index.html). Results (Table 2.13) showed that *M. chelonae* was resistant to amikacin, ceftazidime, meropenem, azithromycin, and trimethoprim/sulfamethoxazole, but was susceptible to tobramycin, and ciprofloxacin. *M. abscessus* was resistant to tobramycin, ceftazidime, azithromycin, meropenem, ciprofloxacin, and trimethoprim/sulfamethoxazole, and was susceptible to only amikacin.

When compared with *M. smegmatis* mc^2155, *M. abscessus* ATCC 19977 had a higher MIC for all agents tested (Table 2.13) except for ceftazidime (both species had MIC >256 μg/ml). *M. chelonae* NCTC 946 had higher MIC values compared to *M. smegmatis* mc^2155 for most antibiotic tested with the exception of azithromycin and ciprofloxacin where MIC values were lower than that of the *M. smegmatis* strain (Table 2.13) and ceftazidime where both species had MIC value >256 μg/ml. Although having slightly different MIC values, based on breakpoint for the agents, the strains of *M. chelonae* and *M. abscessus* had similar susceptibility for most agents with the exception of amikacin, tobramycin, and ciprofloxacin. MIC values for chloramphenicol were 16, 64 and >256 μg/ml for *M. smegmatis*, *M. chelonae*, and *M. abscessus* strains respectively. The results would indicate that *M. chelonae* NCTC 946 and *M. abscessus* ATCC 19977 are much more resistant to antimicrobial agents compared to *M. smegmatis* mc^2155, and that amikacin and tobramycin susceptibility is a good way to separate the two former species (Table 2.13, Figures 2.17-18).

Table 2.13. Antibiotic MICs for mycobacterial species as determined by E-test strips.

Antibiotic	MIC µg/ml (± SD)					
	M. smegmatis mc^{2}155		*M. abscessus* ATCC 19977		*M. chelonae* NCTC 946	
Aminoglycosides						
Amikacin	1 (0)	S	4 (1.1)	S	64 (17.5)	R
Tobramycin	2.5 (0.7)	S	128 (34)	R	12 (3.35)	S
Cephalosporins						
Ceftazidime*	>256 (0)	R	>256 (0)	R	>256 (0)	R
Carbapenems						
Imipenem	,0.75 (0)	S	Nd		Nd	
Meropenem*	1.75 (0.35)	S	>32	R	>32	R
Macrolides						
Azithromycin*	10 (2.83)	R	10 (2.83)	R	4 (1.6)	R
Penicillins						
Piperacillin*	>256 (0)	R	Nd		Nd	
Quinolones						
Ciprofloxacin	0.125 (0)	S	>32	R	0.064 (0.02)	S
Sulfonamides						
Trimethoprim-Sulfamethoxazole (1/19)	0.027 (0.006)	S	>32	R	>32	R
Others						
Chloramphenicol	16 (8)	Nd	>256	Nd	64 (43.1)	Nd

S; susceptible, R; resistant

Susceptibility and resistance are based on breakpoints recommended by NCCLS document M24-T2 (1160), and clinical breakpoints defined by EUCAST http://www.srga.org/eucastwt/MICTAB/index.html.

Nd; not determined.

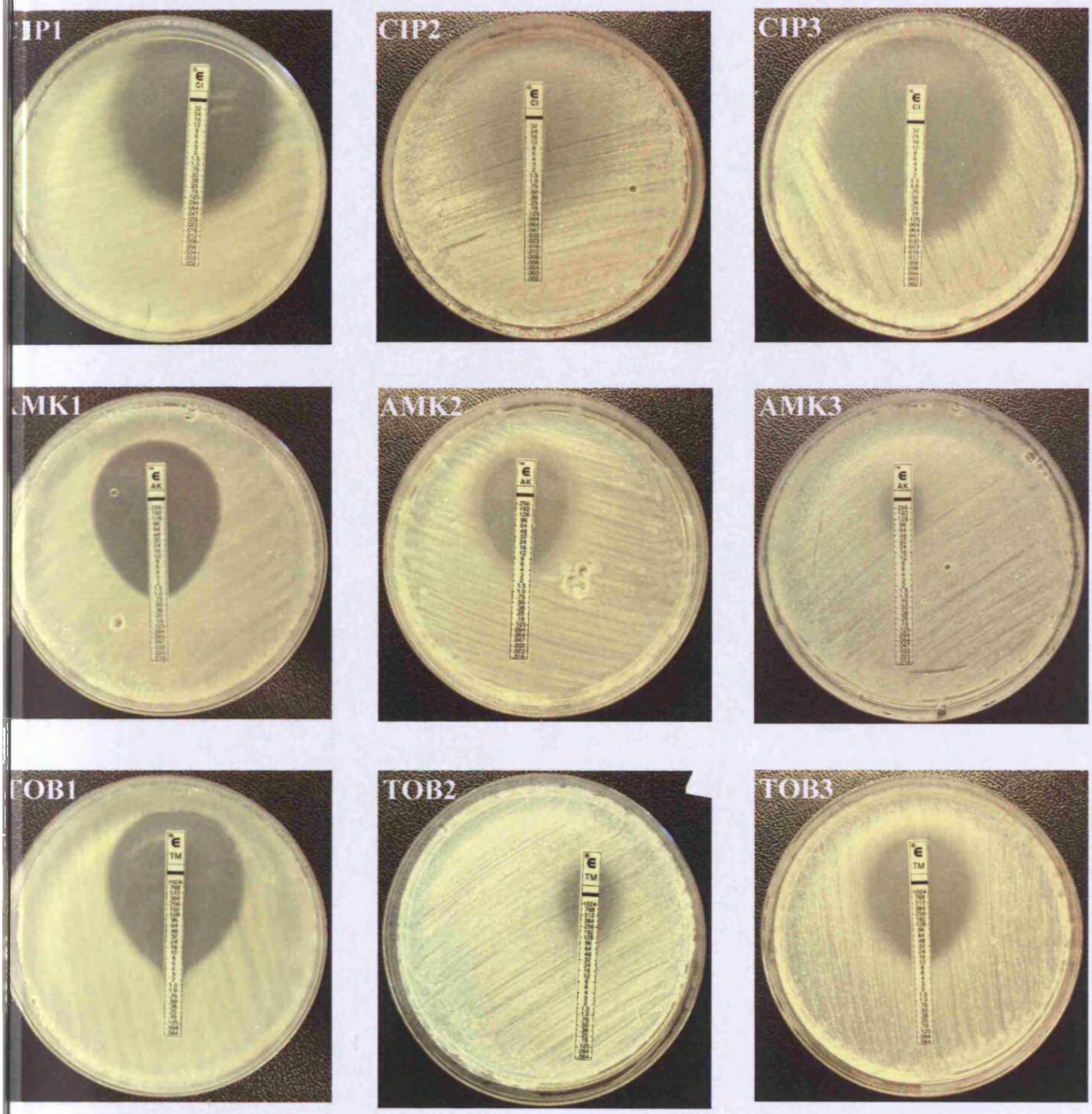


Figure 2.17. Antibiotic susceptibility of mycobacterial species to ciprofloxacin, amikacin, and tobramycin as determine by E-test strips.

MIC values were read after 2 and 4 days incubation at 37^{0}C for *M. smegmatis* and *M. abscessus* respectively, and 4 days incubation at 30^{0}C for *M. chelonae*. All tests were carried out on 7H11 agar.
CIP, ciprofloxacin; AMK, amikacin; TOB, tobramycin; 1, *M. smegmatis* mc^{2}155; 2, *M. abscessus* ATCC 19977; 3, *M. chelonae* NCTC 946.

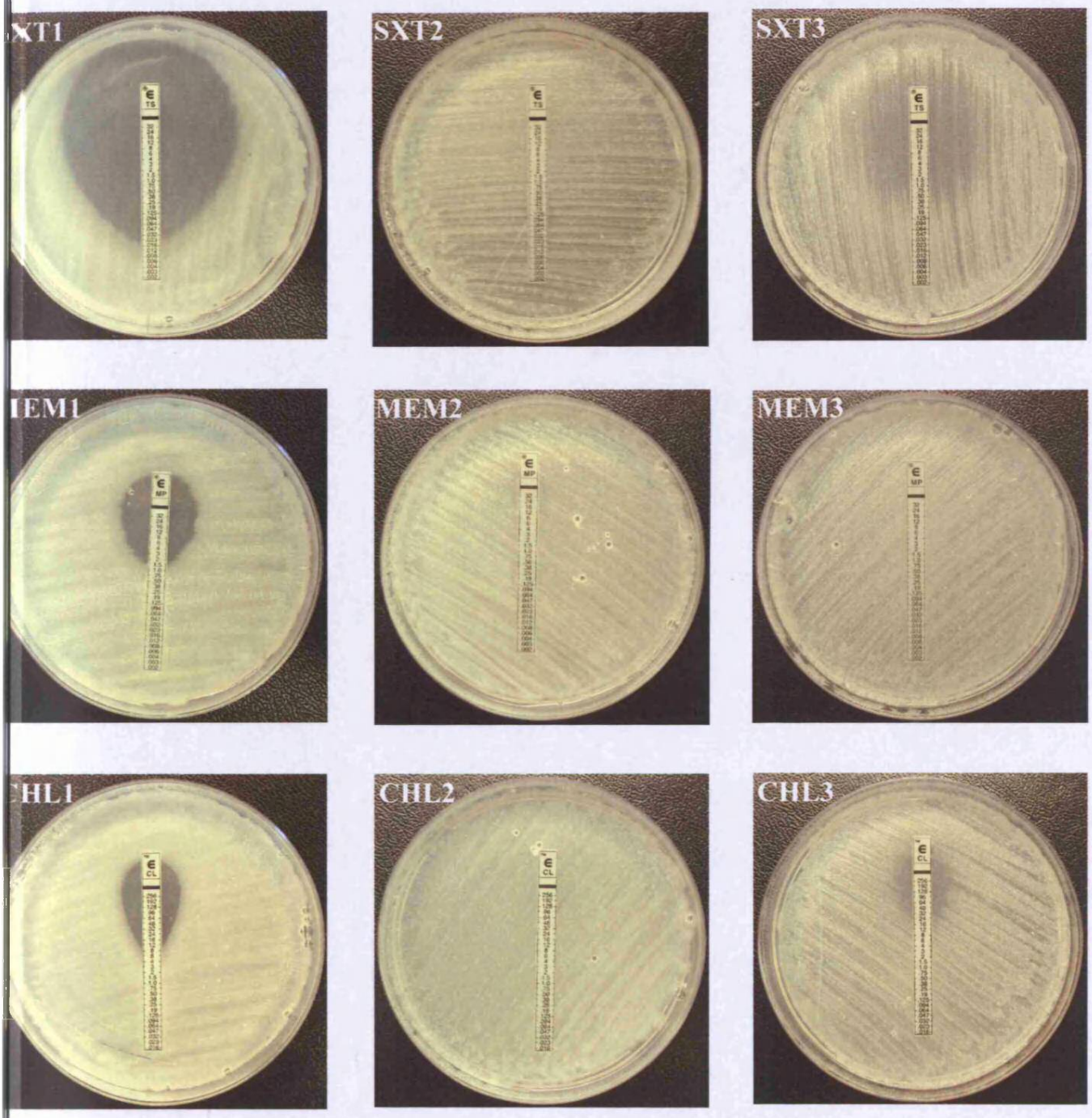


Figure 2.18. Antibiotic susceptibility of mycobacterial species to trimethoprim/sulfamethoxazole, meropenem, and chloramphenicol as determine by E-test strips.

MIC values were read after 2 and 4 days incubation at 37^{0}C for *M. smegmatis* and *M. abscessus* respectively, and 4 days incubation at 30^{0}C for *M. chelonae*. All tests were carried out on 7H11 agar.
SXT, trimethoprim/sulfamethoxazole (1/19); MEM, meropenem; CHL, chloramphenicol; 1, *M. smegmatis* mc^{2}155; 2, *M. abscessus* ATCC 19977; 3, *M. chelonae* NCTC 946.

2.5 DISCUSSION

Growth characteristics and the biocide and antibiotic susceptibility profiles of three rapidly growing mycobacterial species: *M. smegmatis* mc^2155, and *M. chelonae* and *M. abscessus* type strains were described. We also report attempts to isolate *M. smegmatis* mutants with altered biocide sensitivities. Minimal media such as TSB and Sauton were shown to sustain growth of *M. smegmatis* and could be used in routine experiments where richer media such as 7H9 broth are not desired. Testing biocide sensitivity is an example, where organic load is known to interfere with biocide action. Hence, more accurate determination of biocides activity in mycobacteria could be achieved in such minimal media. This was the case in this study, as biocide MICs for all three organisms were shown to be equal to or higher in richer medium than in a minimal one. Therefore we speculate that the actual MICs for these biocides (and probably antibiotics) against the mycobacterial species tested would be lower than those reported.

Natural differences in susceptibility to antimycobacterial agents in the three species tested were noted. These differences occurred even within the closely related *M. chelonae-M. abscessus* group, highlighting the difficulties in adopting routine therapy or disinfection procedures against these organisms. Tobramycin and amikacin were both shown to be effective in distinguishing between the two *M. chelonae* and *M. abscessus* strains. In general terms, the former two strains were more resistant to antimicrobial agents than *M. smegmatis* mc^2155.

One factor that may play a role in this observation is differences in cell wall composition and permeability of these species. The mycobacterial cell wall contains large amount of lipids which, along with the organisation of its components, form an important permeability barrier. *M. chelonae* is one of the most drug-resistant species of mycobacteria, and was shown to be also more resistant to biocides compared to the distantly related *M. smegmatis*. Cell wall characteristics and permeability of *M. chelonae* have been studied by Jarlier and Nikaido (493) and that of *M. smegmatis* by Trais and Benz (1074). Permeation of cephalosporins and small hydrophilic molecules such as

glucose, glycerol, glycine, and leucine across the cell wall was used to determine permeability. It was reported that the permeability coefficients for small hydrophilic solutes were considerably lower in *M. chelonae* and *M. smegmatis* compared to those in Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. For instance in *M. chelonae,* the permeability coefficient of cephalosporins was shown to be 10 and 1000-fold lower than that reported for *P. aeruginosa* and *E. coli* respectively (493). However, differences between mycobacterial species were also noted. Although both *M. smegmatis* and *M. chelonae* were less permeable to cephalosporins than *E. coli* and *P. aeruginosa*, they had different permeability coefficients. These agents crossed the cell wall of *M. smegmatis* 10 times faster than in *M. chelonae*.

Another factor influencing permeability is the presence of porins in the mycobacterial cell wall. These have already been reported in *M. chelonae* and *M. smegmatis* and were shown to have similar properties. However, despite the similarities, the pore diameter of the porin from *M. smegmatis* was around 3 nm, larger than that found in *M. chelonae*, which may also explain differences in permeability (1074).

Sensitivity of *M. smegmatis* mc^2155 to the agents tested could also be explained by the fact that mc^2155 has slightly different cell wall properties and permeability than its parental strain. Etienne *et al.* (274) studied and compared the cell envelope properties of *M. smegmatis* mc^2155 with its parental strain *M. smegmatis* ATCC 607. Investigators showed that the mc^2155 cell envelope lacked polar glycolipids, namely the lipooligosaccharides and the polar subfamilies of glycopeptidolipids. Moreover, there was an apparent difference in the distribution of glycolipids and phospholipids between the outermost and deeper layers of the cell envelope in the two strains. Glycolipids are major lipids in *M. smegmatis* and were shown to significantly contribute to the permeability barrier of the cell envelope of the bacteria (274). Etienne *et al.* (274) reported that the altered nature of the surface-exposed and cell envelope composition in strain mc^2155 was coupled with enhanced permeability. The strain was shown to uptake small hydrophobic molecules much faster than its parent was. This enhanced permeability may also affect sensitivity to both antibiotics and biocides in this strain.

The attempt to isolate *M. smegmatis* mc^2155 mutants with significant alteration in biocide sensitivity was not successful. However, it did show that the *mariner*-transposon system was an effective way for generating random transposon mutants in mycobacteria. The system has already been used to generate transposon mutants in *M. marinum* and *M. smegmatis* (324, 891), and was proven to successfully transpose in other bacteria and protozoa independent of host-specific factors (382, 891). This makes the *mariner* family of transposons a valuable mutagenesis and genetic manipulation tool.

One of the possible reasons for the failure to isolate biocide mutants in this study was the size of the mutant library generated. The preliminary results of the genome sequencing and annotation of *M. smegmatis* mc^2155 show that the genome of the strain is nearly 7 Mbp in size containing nearly 7000 genes. Therefore, a mutant library of 3000 mutants was probably not big enough to cover the whole genome. Nevertheless, the availability of the genome sequence of this organism makes it possible to study genes involved in biocide resistance or susceptibility. Possible ways of achieving this can be as follows.

1) *Using site directed mutagenesis to target genes in M. smegmatis which are already know to be involved in biocide sensitivity in other bacteria.* A number of genes have been reported to affect biocide susceptibility in bacteria (section 1.5). Genes such as the *qac* (*qacG, qacJ, qacA, qacB*), *smr* and *oprR* genes involved in QAC resistance. The *cepA* gene involved in chlorehexidine resistance, the *marA, soxS*, and *accAB* genes for triclosan resistance and the *imp/ostA* gene conferring resistance to GTA. Using targeted mutagenesis to inactivate these genes or their homologues in *M. smegmatis*, would be a good way to test for their role in biocide resistance in this organism. An example is the *M. tuberculosis* H37Rv *efpA* gene, which is also present *in M. leprae, M. bovis, M. avium* and *M. intracellular* and encodes a putative efflux protein EfpA. The latter was shown to have a similar secondary structure to Pur8, MmrA, TcmA, LfrA, EmrB, and other members of the QacA transporter family (QacA TF) which mediate antibiotic and QACs resistance in bacteria (242). The *efpA* gene has been detected by Southern

hybridization in a number of rapidly growing mycobacterial species including *M. smegmatis* (242). Hence, this gene may play a role in biocides, namely QACs, resistance in this organism, and targeting it using site directed mutagenesis would be a good way to test this hypothesis.

2) *Targeting genes involved in antibiotic resistance in mycobacteria or other bacteria.* The genetic basis of antibiotic resistance in bacteria has been well studied, and a large number of genes have been identified that confer increase susceptibility or resistance to these agents including in mycobacteria (section 2.1.7.4.3). Because of some similarities in the way antimicrobial agents enter and affect the cells, it is possible that genes conferring resistance to antibiotics will also affect biocide sensitivity (94). This has been shown numerous times, whereby biocide sensitive mutants in both mycobacteria and other bacteria have also expressed altered antibiotic susceptibility profiles. For instance, the *inhA* mutation in *M. smegmatis* also conferred isoniazid resistance (683). In addition, the expression of the *E. coli* multiple antibiotic resistance *marA* gene (also involved in triclosan resistance) in *M. smegmatis* produced increased resistance to multiple antimicrobial agents, including rifampin, isoniazid, ethambutol, tetracycline, and chloramphenicol (675). Hence targeting antibiotic resistance genes or their homologues, or screening the already isolated antibiotic sensitive or resistant mutants in *M. smegmatis* (Table 2.3) would be a good way to identify genes involved in biocide sensitivity.

3) *Targeting genes that may influence biocide sensitivity.* The sequencing of the *M. smegmatis* genome revealed that the bacterium has up to 7000 genes, and nearly 5000 of these have already been assigned functions. It is therefore possible to target genes with functions that may be involved in biocide sensitivity. For instance, genes involved in biosynthesis of cell wall components such as, the peptidoglycan, mycolic acids or other lipids. The *inhA* gene in *M. smegmatis* involved in lipid biosynthesis and conferring triclosan resistance is a good example. Some genes involved in cell wall biosynthesis have already been

identified by the genome sequencing project including genes encoding the mycolic acid synthesis protein "methoxy mycolic acid synthase 1", the peptidoglycan pathway proteins such as the "UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase" and "phosphoglucosamine mutase (GlmM)", and surface polysaccharides and lipopolysaccharides biosynthesis proteins such as "cyclopropane-fatty-acyl-phospholipid synthase 1" and "acetyl-coenzyme A synthetase". Because of their role in cell wall biosynthesis and the importance of the latter as a permeability barrier in mycobacteria, we can speculate that inactivation of some of these genes in *M. smegmatis,* may cause changes in antimicrobial sensitivities.

Other possible target genes include genes encoding cell membrane transporters, porins, milti-drug resistance proteins, and efflux pump systems. A porin has already been identified in *M. smegmatis* (734), and from the genome sequencing, a number of drug resistance proteins, efflux pumps and transporters have been identified. These include ABC-type multidrug transporter systems, SMR family multidrug resistance proteins, MFS transporters including the Bcr/CflA subfamily drug transporters, membrane permeases and transporters (e.g. YgbN, YdfJ, UspE, YddQ, SfuB, YjfF, YddQ, YphF, RutG), membrane proteins (e.g. MmpS1, MmpL) and lipoproteins (e.g. Lpps, YaeC, YaeQ, nlpa, MK35).

Although we can only speculate about the involvement of some of the above proteins in biocide resistance in mycobacteria, it is a fact that resistance to many antimicrobial agents including biocides have been linked to genes encoding proteins with some of the above functions (683). Moreover, examples exist in mycobacteria themselves, for instance the *inhA* gene in *M. smegmatis* involved in fatty acid synthesis and conferring triclosan resistance when deactivated (683). The *lfrA* gene encoding a membrane efflux pump of the MSF family in *M. smegmatis* (1045) is another example. The gene has been shown to be involved in resistance of mycobacteria to a number of antimicrobial agents, including several quinolones (617, 939). In *M. smegmatis* disruption of the *lfrA* gene rendered the mutant more susceptible to

ethidium bromide, acriflavine, ciprofloxacin, doxorubicin, and rhodamine 123 (two- to eightfold decrease in MICs) (939). The *lfrA* gene is homologous to *qacA* from *S. aureus* which has already been shown to be involved in QACs resistance (798, 804, 887), and appeared to recognize some QACs in *M. smegmatis* (1045). Hence, we can speculate that this gene may play a role in QAC resistance in this organism.

2.6 CONCLUSION

- The mycobacterial strains tested had different biocide and antibiotic susceptibilities, with *M. smegmatis* mc^2155 being more susceptible to antimicrobial agents than the *M. chelonae* or *M. abscessus* type strains.

- Chlorhexidine diacetate was the most effective biocide at inhibiting growth of the mycobacterial strains tested.

- Low concentrations of chlorhexidine diacetate, cetylpyridinium chloride, and triclosan were mycobacteriostatic. However, high concentrations of *ortho*-phthalaldehyde were required to inhibit growth.

- Based on agar MICs, chlorhexidine diacetate, cetylpyridinium chloride, and triclosan at the "in-use" concentrations are all effective at inhibiting growth of *M. smegmatis* mc^2155.

- Chlorhexidine diacetate, and triclosan, at the "in-use" concentrations are also effective at inhibiting growth of *M. chelonae* NCTC 946 and *M. abscessus* ATCC 19977.

- The "in-use" concentration of *ortho*-phthalaldehyde appears to be not effective at inhibiting growth of the mycobacterial strains tested. However, it is important to note that the killing concentrations rather than agar MICs are the main measure of biocide effectiveness. In this context, a 0.5% concentration of OPA (lower than its "in-use" concentration) was shown to be rapidly mycobactericidal against a range of NTM and more importantly against GTA-resistant mycobacterial strains (311).

- Transposon mutagenesis using a *mariner*-based system was not successful at isolating *M. smegmatis* mc^2155 mutants with altered biocide susceptibility.

CHAPTER III

MOLECULAR BASIS OF BIOCIDE RESISTANCE AND SUSCEPTIBILITY IN SERRATIA

ABSTRACT

Serratia marcescens is a problematic opportunistic pathogen that frequently causes nosocomial infections due to it ability to contaminate solutions or devices. *S. marcescens* resistance and susceptibility to antibiotics is well known, however, very little is known about the mechanisms of *Serratia* resistance and susceptibility to biocides. Agents such as triclosan (TRI), chlorhexidine diacetate (CHX), quaternary ammonium compounds and aldehydes are widely used in the home and clinical settings as a mean to disinfect instruments and clean surfaces. This study aimed to identify the molecular basis of biocide resistance and susceptibility in *S. marcescens* Db11, a model strain for which the complete genome sequence is available. The strain is non-pigmented, grew well overnight at 37^0C on TSA and TSB and was shown to have a typical bacterial growth curve. The growth inhibitory and killing effects of four biocides; TRI, cetylpyridinium chloride (CPC), CHX and alkaline *ortho*-phthalaldehyde (OPA) on *S. marcescens* Db11 were investigated. From the Minimal Inhibitory Concentration (MICs) of the biocides it was shown that CHX and CPC were most effective at inhibiting bacterial growth with agar MIC ranges of 16-20 μg/ml and 90-110 μg/ml respectively. TRI and OPA demonstrated less inhibition, possessing MIC values of 3800-4000 μg/ml and 2800-3200 μg/ml, respectively. Antibiotic susceptibility profile for *S. marcescens* Db11 was also determined and showed that the strain was resistant to amikacin, tobramycin, chloramphenicol and azithromycin, but susceptible to ceftazidime, ciprofloxacin, imipenem, piperacillin, and meropenem. To determine the genes involved in susceptibility to the four biocides, 6000 *S. marcescens* Db11 random transposon mutants (generated using a mini-Tn*5*Km2 transposon system) were screened on agar containing a concentration of biocide just below its respective MIC. Eighty mutants showed varying degrees of sensitivity to at least one of the four biocides, 26 of these were further investigated along with two control mutants. The mutants did not show major changes in biocide susceptibility as indicated by agar MIC values. However, the changes were shown to be reproducible, and were confirmed by results of other tests notably broth MICs, lethality and potassium leakage tests. The genetic basis of 24 mutants was determined and demonstrated putative mutations in genes encoding proteins with varying functions. These included anabolism and catabolism, gene regulation, cell envelope biosynthesis, porin, energy production, and virulence. A number of the disturbed genes were in close proximity to other genes encoding regulatory proteins, membrane transporters, antimicrobial resistance efflux proteins, and proteins with other important cellular functions. Mutational polar effect in some of the disrupted genes may have contributed to the mutants' observed phenotype. Two mutants, one deficient in the outer membrane protein A (OmpA), and another deficient in the nucleoid-associated protein (NdpA), were chosen for complementation analysis. Complementation of the *ndpA* mutant which showed increased resistance to CPC and CHX but was sensitive to TRI, lead to restoration of the wild type phenotype. Complementation of the *ompA* mutant, which showed multiple sensitivity to CHX, TRI and OPA however, did not restore the wild type phenotype as the cloned *ompA* gene was shown to be transcribed but not translated in the complemented mutant. In summary, the genetic basis for biocide resistance in *S. marcescens* Db11 is multi-factorial and encoded by several novel loci worthy of further study.

3.1 INTRODUCTION

3.1.1 Taxonomy of the genus *Serratia*

The genus *Serratia* belongs to the family *Enterobacteriaceae* which includes other well known genera such as *Escherichia*, *Shigella*, *Salmonella* and *Yersinia*. Many species of *Serratia* produce a non-diffusible, water-insoluble red pigment, prodigiosin (2-methyl-3-pentyl-6-methoxyprodiginine). The earliest manifestation of the organism was traced by many authors (328, 402) back to antiquity as blood-like drops on pieces of bread. In 1823 Bizio (80) demonstrated that these blood-like manifestations were due to the development of a microorganism which he named "*Serratia marcescens*". A year later, Sette (969) also linked the blood-like spots on food to an organism which he named *Zaogalactina imetrofa*. Unfortunately the red-pigmented microorganisms studied by Bizio and Sette were not preserved. Working in Germany on the same phenomenon, Ehrenberg (257) isolated a motile organism which he named *Monas prodigiosa* and he concluded that this organism was the cause of the red spots on food. Unfortunately, Ehrenberg's strain was also not preserved and the identity of *M. prodigiosa* is uncertain. In 1884, R. Koch isolated a red-pigmented bacterium from the digestive tract of a monkey in India. The strain was preserved (ATCC 4002) and named *Bacillus indicus* (259). Since then a large number of red-pigment producing organisms have been isolated and named. However, it was not until early 1900s when Hefferan classified rod-shaped red-pigment nomenspecies into 4 groups one of which was the prodigiosus group which correlated to red-pigmented enterobacteria (417).

The first generic name for a recognizable red-pigmented enterobacterium was *Erythrobacillus* given by Fortineau in 1904 (300). However, the nomenclature used in *Bergey's Manual* (68) and other authors imposed the use of the generic name *Serratia.* In the same manual, *Serratia,* the sole genus in the tribe *Serrateae*, was included in the family *Enterobacteriaceae* (99). From the first to the seventh editions of *Bergey's Manual,* the number of species in the genus *Serratia* dropped from 23 to 5. These five species were *S. marcescens, S. indica, S. plyrauthicum, S. kilensis*, and *S. piscatorum.*

The taxonomic status of *Serratia* was still unclear with species being rejected or reestablished into the genus. In 1957, Davis *et al.* (220) suggested that the genus *Serratia* should include only one species *S. marcescens*. This concept of mono-specified genus *Serratia* was almost universally accepted in the 1960s even after the inclusion of the genus into the tribe *Klebsielleae* in 1962 (275). Later, taxonomic work demonstrated the existence of at least 4 species in the genus *Serratia* with different biotypes: *S. marcescens, S. liquefaciens, S. plymuthica,* and *S. marinorubra.* Currently, the taxonomic information recognizes the following species within the *Serratia* genus: *S. ficaria, S. fonticola, S. grimesii, S. liquefaciens, S. marcescens, S. marinorubra, S. entomophila, S. odorifera, S. plymuthica, S. proteamaculans, S. quinivorans, S. rubidaea,* and *S. ureilytica.* Some of these species such as *S. liquefaciens* form complexes.

3.1.2 Characteristics, habitats and clinical significance of the genus *Serratia*

3.1.2.1 Characteristics of the genus *Serratia*

Serratia are Gram-negative, facultative anaerobic rods ranging from 0.3 to 1.0 μm in width to 0.6 to 6.0 μm in length. Like other *Enterobacteriaceae, Serratia* grow well on ordinary media under anaerobic and aerobic conditions. While some strains of *S. plymuthica* may not grow at 37^{0}C, most members of this genus grow well between 25 and 37^{0}C. Growth is inhibited at temperatures above 45^{0}C, while variable growth has been observed at 5^{0}C and 40^{0}C. Optimal pH for growth of these organisms has been shown at pH 9, and they are inhibited at pH less than 4. Colonies of *Serratia* have long been known to produce red pigment. However, pigmentation is observed in only small percentage of isolated cultures and it is variable depending on species and other factors such as incubation period. Three species are known to produce the red pigment prodigiosin, and they include some strains of *S. marcescens* and most strains of *S. rubidaea* and *S. plymuthica.* Grimont *et al.* (371) however, also reported that strains of *S. marinorubra* also produced the pigment.

Biochemical characteristics of *Serratia* species have been well studied and are used to differentiate between species within the genus and the family. In 1977 Grimont *et al.* (371) undertook a large study on 156 isolates of *Serratia* and related bacteria including representatives of *Enterobacter* and *Erwinia*, using 223 morphological, physiological, biochemical and carbon source utilization tests. The results of the study determine some of the basic characteristics of species in the genus *Serratia* (Table 3.1) and the differences between this genus and other closely related organisms. Grimont *et al.* (371) reported that all the bacteria studied were catalase positive, utilized glucose by fermentation, and utilized *N*-acetylglucosamine, aspartate citrate, D-fructose, gluconate, D-glucose, L-malate, mannitol and D-ribose as sole carbon sources. Investigators also reported that all strains studied were negative for growth at pH 4, phenylalanine deaminase, urease, and for growth on a number of compounds as sole carbon source (371). Along with the production of red pigment, production of special enzymes by species of *Serratia* has been used to differentiate the genus from other closely related *Enterobacteriaceae,* especially *Enterobacter*. Examples of such enzymes include chitinase, DNAse, esterase, gelatinase, lecithinase, and lipase.

3.1.2.2 Habitats of the genus *Serratia*

Members of the genus *Serratia* are very widely spread and have been isolated from soil, air, water, plants, insects, animals and humans. Because of the taxonomic confusion regarding this genus, early ecological information is not clear. Red-pigmented *Serratia* are able to be traced back to early publications if they have been preserved, whereas non-pigmented species are difficult to trace even in more recent work. Red-pigmented gram-negative bacilli isolated from air, water, and soil have been recorded as early as the 1900s (639, 710). Members of the *Serratia* genus have been isolated from both residual (938) and seawater (372). *Serratia* occurs naturally in soil, and strains of soil origin have appeared in a number of studies (21, 204, 491, 530).

Serratia associated with plants have also been reported (66, 368, 374, 513, 1162), and its origins are thought to be the soil, although there are exceptions such as the case of

Serratia associated with figs of the Calimyrna variety, where it is of insect source (1016). In the course of an ecological survey, Grimont *et al.* (370) isolated *Serratia* from number of plant species including: *Eucalyptus, Pistacia,* bitter cherry, *Acacia,* coconuts, sorghum, grass, mushrooms, tomatoes, leeks, green onions, brussels sprouts, lettuce, broccoli, artichokes, radish, spinach, carrots, and figs. *Serratia* are often associated with insects of many orders including Orthoptera, Isopteran, Coleopteran, Lepidoptera, Hymenoptera, and Diptera (370). *Serratia* species frequently have been recovered from healthy, diseased, or dead insects (124, 125, 635, 1010) and more than 70 species of insects were found susceptible to *Serratia* infections (124, 370, 485, 508, 832, 1009, 1011, 1128). Association of *Serratia* with animals has been well documented (370). The organism was isolated from cold blooded vertebrates (138, 370, 484) as well as from both wild and domesticated animals (47, 90, 370, 453, 514, 888, 1150, 1185). Species of *Serratia* exist in the organs and intestines of fish (54, 659), and *S. liquefaciens* is considered a fish pathogen reported to have caused infection in Atlantic salmon populations (680), in juvenile cultured rainbow trout (25), and in turbot (1093).

Table 3.1. Biochemical characteristics of members of the genus *Serratia.* Adapted and modified from Abbot (1).

Species	Production of:		Mal utilization	Acid production from:								Red pigment	Odour
	LDC	ODC		Ara	Rha	Xyl	Suc	Adon	Sorb	Cello	Arab		
S. entomophila	-	-	-	-	-	V	+	-	-	-	V	-	-
S. ficaria	-	-	-	+	V	+	+	-	+	+	+	-	V
S. fonticola	+	+	V	+	V	V	V	+	+	-	+	-	-
S. liquefaciens	+	+	-	+	V	+	+	-	+	-	-	-	-
S. marcescens	+	+	-	-	-	-	+	V	+	-	-	V	-
S. marcescens biogroup 1	V	+	-	-	-	-	+	V	+	-	-	NA	-
S. odorifera biogroup1	+	+	-	+	+	+	+	V	+	+	-	-	+
S. odorifera biogroup2	+	-	-	+	+	+	-	V	+	+	-	-	+
S. plymuthica	-	-	-	+	-	+	+	-	V	V	-	+	-
S. rubidaea	V	-	+	+	-	+	+	+	-	+	V	+	-

LDC; lysine decarboxylase, ODC; ornithine decarboxylase, Ara; arabinose, Mal; malonate, Rha; rhamnose, Xyl; xylose, Suc; sucrose, Adon; adonitol, Sorb; sorbitol, Cello; cellobiose, Arab; arabitol, +; ≥90% of strains, V; 10-90% of strains, -; ≤10% of strains, NA; not available.

3.1.2.3 Clinical significance of the genus *Serratia*

A healthy human has little chance of being infected by *Serratia*, which was long considered a normal commensal of the intestine and a saprophyte, and used as the indicator bacteria in studies to trace bacterial transmission and penetration in man (556, 839). Nowadays the organism is known to be an important opportunistic pathogen, of which *S. marcescens* is the most common species associated with human diseases, followed by strains of the *S. liquefaciens* complex (368). Grimont and Grimont (370) mentioned that no significant infections due to *S. plymuthica* have been reported. Also, *S. marinorubra* represented less than 4% of *Serratia* isolated from human samples (372, 416). Strains of the *S. liquefaciens* complex have also been isolated from human clinical specimens (86, 368), and are known to predominantly cause sepsis and bloodstream infections via contaminated clinical equipment and blood component (89, 251, 271, 376, 400, 699, 968).

S. marcescens is undoubtedly the most common *Serratia* associated with human diseases, and is the only named species of the genus that is really nosocomial (368). The pathogenicity of this organism to humans was first noted in 1913 where it was reported to be the cause of a pulmonary infection (1161). However, the prevalence of *S. marcescens* in human diseases had been underestimated for years before the first known outbreak of nosocomial *S. marcescens* infection in 11 cases at Stanford University Hospital was reported (1139). Since then, infections with the organism have been noted with increasing frequency. Taxonomic studies have shown that non-pigmented *S. marcescens* belong to biotypes and serotypes that are different from pigmented *S. marcescens* and that non-pigmented *S. marcescens* are the most important in hospital infections (368). Moreover, the non-pigmented *S. marcescens* are usually more resistant to antibiotics than the pigmented isolates which are rarely responsible for hospital outbreaks (23).

S. marcescens possesses a number of virulence factors that allow it to be an important opportunistic pathogen. One aspect of this pathogenicity is the ability of this organism to adhere to the target tissue surface. Adhesion to epithelial cells mediated by the type 1

fimbriae of *S. marcescens* has been described (599, 1175), and the fimbriae of *S. marcescens* have been shown to contribute to superoxide production (661) and phagocytosis (717). *S. marcescens* lipopolysaccharides are also important for pathogenicity and virulence, and are crucial for the biological activity of endotoxins. Palmora *et al.* (785) showed that bactericidal action on *S. marcescens* depended upon the O-side chain length of lipopolysaccharides. In addition, the organism has been reported to produce a number enzymes involved in pathogenicity including proteases, nucleases, lipases, chloroperoxidase, a chitinase, a lecitinase and a hemolysin (425) and cytotoxin (139).

As mentioned above *S. marcescens* is a notorious nosocomial opportunistic pathogen and its predominant mode of transmission is from person to person. Other modes of transmission include spread through various contaminated medical apparatuses, intravenous fluids, and clinical solutions. These solutions included saline in plastic bottles (673), water in ultrasonic nebulisers (871, 882), solutions used for inhalational treatment (694, 940), quaternary ammonium disinfectant solutions (258) and hand lotions (727). The ability of *S. marcescens* to cause infections was once thought to be limited to patients with chronic debilitating disorders. However, the organism has now been implicated in very diverse kinds of human infections.

S. marcescens is often involved in respiratory tract colonization and infection in nurseries and patients in intensive care units (22, 134, 674, 694, 1013), as well as in urinary tract infections especially in patients with indwelling catheters (580, 648). Some of these outbreaks can be prolonged, lasting more than a year (424, 630, 779, 926). Infection or superinfection of surgical wounds is another commonly reported *S. marcescens* manifestation (134, 1145), and can lead to the development of *S. marcescens* septicemia which is frequently fatal (1145). Other reported cases of *S. marcescens* septicemia include a case of nosocomial epidemic of *S. marcescens* septicemia ascribed to contaminated blood transfusion bags (426), an epidemic outbreak in a hemodialysis unit (813), following artificial kidney dialysis (270), posttransfusion (443) and post-abortion (287) cases, and even a case in a previously healthy woman have been reported (224).

Occasionally, *S. marcescens* is associated with meningitis (368, 811) which can be fatal (496), brain abscesses (368, 934), bullous cellulites (191), and conjunctivitis (687). Treatment of infections caused by *S. marcescens* may be difficult because the organism is resistant to a variety of antibiotics including ampicillin and both first and second generation cephalosporins (38), adaptive antibiotic resistance may also develop (section 3.1.5).

3.1.3 Genetics of *Serratia*

Early studies on *Serratia* genetics were carried out by Laurent (585) who found in 1890 that UV rays from the sun induced non-pigmented mutants of the Kiel bacillus. Since then molecular methods have been used extensively in determining the taxonomic status of the genus, and have advanced studies of virulence and quorum sensing (section 3.1.4). The following is an overview of the *Serratia* genome and its G+C content, transduction, and plasmids and conjugation in this genus.

The G+C content of *Serratia* DNAs is usually given as 54-60 % (187, 437), and the 59.51% G+C content of the recently sequences genome of *S. marcescens* strain Db11 is a good example. The range of G+C content of DNA for a number of *Serratia* species was determined as follows: *S. marcescens,* 57.5-60.4% (average around 58%); *S. liquefaciens,* 52.6-54.4%; *S. plymuthica,* 53.3-56.3%; and *S. marinorubra,* 53.5-58.3% (187, 371, 437, 653). Gillis *et al.* (349) determined the genome size of one strain of *S. marcescens* and found it to be 3.57×10^9 Da with a C+G content of 59.9%. Grimont and Grimont (370) reported that *S. marcescens* DNA has the highest G+C content among enteric bacteria and that compared to the genome of *E. coli* K12, the genome of *S. marcescens* is larger by 10^9 Da. They concluded that these differences in size and composition of DNA between *S. marcescens* and *E. coli* suggest that the enzymatic regulations and the genetics of *S. marcescens* might be different from those of *E. coli.*

Recently the genome sequencing of *S. marcescens* Db11 was completed at the Sanger institute (http://www.sanger.ac.uk/Projects/S_marcescens/), and results revealed that the

genome consists of a single circular chromosome of 5,113,802 bp with a G+C content of 59.51%. The isolation of the non-pigmented *S. marcescens* Db11 strain, (Db for *Drosophila* bacterium) was first reported by Flyg *et al.* (298) during their investigation into the insect pathogenic properties of the species. Flyg and colleagues isolated *S. marcescens* strain Db10 from moribund *Drosophila melanogaster* strain 153, from which a spontaneous mutant Db11 which was resistant to 100 μg/ml streptomycin was isolated (298). Strain Db11 was shown to have similar pathogenicity as the parental Db10 and was lethal to *Drosophila* when given in the food or by injection (298).

Transduction in *Serratia* was demonstrated as early as 1956 when Belser & Bunting (61) described genetic transfer between auxotrophic mutants of *S. marcescens* HY that was suggestive of a transduction mechanism. Although the HY strain had not been shown to be lysogenic, some mutants of the strain were shown later to produce a phage (phage y) (516). A second transduction system that used *S. marcescens* HY and phage kappa (κ) was described by Steiger *et al.* (1007), and using both systems (phages y and κ), 18 auxotrophy genes were mapped in this bacterium (1003). In 1973, a generalized transduction system was studied with *S. marcescens* Sr 41 and phage PS 20 (666, 667). In 1991, Regue *et al.* (862) reported another generalized transducing phage in *S. marcescens*, Φ3M, belonging to the Myoviridae family. Until recently, no transducing phage capable of infecting *S. marcescens* Db11 had been found. However, in 2006, Petty *et al.* (823) reported the isolation and characterisation of a likely virulence phage ΦIF3 capable of mediating generalized transduction in *S. marcescens* Db11. Investigators concluded that this transduction system would be a valuable tool for functional genomic analysis of the host.

Plasmids and conjugation have also been described in *Serratia.* Grimont and Grimont (370) noted that no chromosomal transfer via conjugation was found in any species of the genus *Serratia.* Attempts at chromosomal conjugation from Hfr strains of *E. coli* to *S. marcescens* were reported to be unsuccessful or limited to the transfer of "F factors" without integration in *S. marcescens* chromosome (279, 937). Different types of plasmids have been reported in *Serratia* (415, 597), although most work was concentrated on *S.*

marcescens. Plasmids found in *Serratia* include metabolic plasmids demonstrated in *S. liquefaciens* (587), plasmid pCP-1 reported in *S. plymuthica* (1067), and multiple resistance plasmids which are especially common in nosocomial strains of *S. marcescens* (416, 951). According to Grimont and Grimont (370), six plasmid incompatibility groups (L, S, C, M, P, and F_{II}) have been shown in *S. marcescens*, with the L group found only in *S. marcescens*.

R plasmids are common in nosocomial strains of *Serratia* conferring resistance phenotype to antibiotics and toxic heavy metals. Examples of these include a plasmid conferring resistance to aminoglycosides reported in *S. marcescens* S-95 (239) and the R478 plasmid isolated from a clinical isolate of *S. marcescens* (691) and shown to confer resistance to tetracycline, chloramphenicol, kanamycin, mercury, silver, copper, arsenic, and tellurite (350). R plasmids are usually detected by their transferability to *E. coli* K12, and although plasmid transfer from *Serratia* to *E. coli* is considered inefficient (416), such transfer has been described (369, 416, 598, 950, 951). Platt and Sommerville (830) described a simple conjugation system for the transfer of plasmids from *Serratia* species to members of the genus *Enterobacter*.

3.1.4 Quorum sensing in the genus *Serratia*

3.1.4.1 General overview

Microorganisms are consistently subjected to environmental stimuli including changes in temperature, osmolarity, pH and nutrient availability. In response, bacteria have developed sophisticated mechanisms that sense, gather, process, and transduce environmental fluctuations, which allow adaptation to these changes (794). One such mechanism is "quorum sensing" (320), the process of cell-to-cell communication via the use of small signalling molecules termed "autoinducers" or "pheromones. The extracellular concentration of these molecules is related to the population density of the producing organism. They can be sensed by cells and this allows the whole population to initiate a concerted action once a critical concentration (corresponding to a particular cell

density) has been achieved (1088, 1142). There is a vast assortment of different classes of chemical signals employed in quorum sensing (51, 297, 444, 449, 952, 1025), of which the *N*-acyl-homoserine lactones (AHLs) are the most commonly used in Gram-negative bacteria (586). Individual species of bacteria can use more than one chemical signal and/or more than one type of signal to communicate.

Quorum sensing signaling systems control diverse physiological functions in gram-negative and gram-positive bacteria. Few examples are bioluminescence in *V. fischeri* (749), biofilm formation in *P. aeruginosa* and *S. gordonii* (218, 686), conjugal transfer of the Ti plasmids from *Agrobacterium tumefaciens* (1194), production of extracellular cell-wall-degrading enzymes in *Erwinia carotovora* (828), production of antibiotics in *E. carotova*, *Photorhabdus luminescens* and *Streptomyces coelicolor* (34, 234, 1040), induction of virulence factors in *P. aeruginosa* (584, 809), competence in *S. pneumoniae* (405), and competence and sporulation in *B. subtilis* (1065).

3.1.4.2 Quorum sensing in *Serratia*

Quorum sensing in *Serratia* has been identified and shown to control a wide range of biological and ecological functions, and often interconnect with other global regulators. It was reported to command swarming and sliding motility in *S. marcescens* MG1 (256) and *S. marcescens* SS-11 (450) respectively, as well as biosurfactant production in the latter (450). Quorum sensing directs the expression of the LipB protein translocation system in *S. marcescens* MG1 and *S. proteamaculans* B5a (164, 879), as well as the production of a number of extracellular enzymes. These include protease, chitinase and haemolytic activity in *S. marcescens* strain 12 (196), nuclease in *S. marcescens* SS-1 (450), nuclease, chitinase and protease in *S. plymuthica* RVH1 (1088), and pectate lyase and cellulase in *Serratia* sp. ATCC 39006 (989, 1059). The production of prodigiosin (373), a reddish pigment described as a secondary metabolite (122) with a number of biological properties including antibacterial, antimalarial, antifungal and antiprotozoal activities (229, 1147), as well as potent immunosuppressive, proapoptic and anticancer properties (64, 654, 815, 816), is also controlled by quorum sensing (450, 989, 1059).

The latter also directs the production of antibiotics in a number of *Serratia* species including *S. plymuthica* RVH1 (1088), *S. marcescens* strain 12 (196), and *Serratia* sp. ATCC 39006 (678, 679). This form of cell-to-cell communication has also been reported to control biofilm formation in *S. marcescens* MG1 (255, 575, 872).

3.1.4.3 Quorum sensing in pathogenic bacteria, a potential antimicrobial target?

Because quorum sensing regulates an array of biological functions including virulence factors in a number of pathogenic bacteria, it is considered as an ideal target for inhibition of infections by "antipathogenic" drugs which specifically inhibit bacterial virulence rather than kill or inactivate organisms (12). The principle of this approach is that interference with cell-cell signaling by a small molecule antagonist (quorum-sensing blocker), which competes for the signal molecule-binding site of sensor or transcriptional activator proteins would switch off virulence gene expression and so attenuate the pathogen (292). A number of quorum-sensing blockers have been reported (254, 353, 445, 672, 1037, 1055), and promising results have been demonstrated in *P. aeruginosa* (429, 430) and *S. aureus* (669).

3.1.5 *Serratia* and antimicrobial agents

3.1.5.1 Antibiotic resistance in *S. marcescens*

S. marcescens is naturally resistant to a many antibiotics, a large number of which are carried on multi-resistance R-plasmids. These plasmids are found almost exclusively in non-pigmented biotypes, and the lack of antibiotic resistance plasmids in pigmented *S. marcescens* has been reported (327). In 1968, Medeiros and O'brien (416) studied the contribution of the R-factors to the multiple antibiotic resistance of *Serratia.* They reported that multiple drug resistance is more prevalent in *Serratia* than in any other commonly isolated member of the *Enterobacteriaceae*, and that the R-factors not only mediated resistance to drugs to which the strain was previously susceptible but also conferred additional degrees of resistance to drugs to which the organism was already

resistant. Investigators also found that 95% of the multi-resistant isolates they studied transferred at least part of their resistance (416).

Stock *et al.* (1022) studied the natural susceptibility of 77 strains of *S. marcescens* and 41 strains of the *S. liquefaciens* complex, to 70 antibiotics using micro-dilution procedure. They reported that all species were naturally resistant to benzylpenicillin, oxacillin, cefaclor, cefazolin, cefuroxime, numerous macrolides including erythromycin, roxithromycin and clarithromycin, lincosamides, streptogramins, glycopeptides, rifampicin and fusidic acid. Uniform natural sensitivity was found to most aminoglycosides, several acylureidopenicillins, ticarcillin, newer cephalosporins, carbapenems, aztreonam, quinolones and antifolates (1022). Differences in susceptibility to antibiotics between the *Serratia* species were also noted. For instance, in contrast to species of the *S. liquefaciens* group which were sensitive to all aminoglycosides, *S. marcescens* was naturally sensitive and intermediate to netilmicin and tobramycin and less susceptible than *S. liquefaciens* to amikacin and ribostamycin. Moreover, *S. marcescens* was the only species that was uniformly naturally resistant to tetracycline, amoxycillin, amoxycillin/clavulanate and loracarbef (1022). *S. marcescens* is also known to be highly resistant to polymyxin B. In a survey of 95 strains of *S. marcescens*, Wilfert *et al.* (1144) reported that all strains were not inhibited by a concentration of 100 μg/ml of polymyxin B. They were uniformly sensitive to gentamicin, and an apparent resistance to kanamycin and nalidixic acid among endemic strains was also noted. Antibiotic multi-resistant strains of *S. marcescens* have also been reported (1026).

3.1.5.1.1 Resistance to β-lactam antibiotics

Resistance to β-lactams in many *Enterobacteriaceae* including *Serratia* arises by a number of mechanisms. These include high level production of chromosomal Ambler class C-type cephalosporinase (565), acquisition of an Ambler class A extended-spectrum β-lactamase (1177), acquisition of metallo-β-lactamases (1177), decreased permeability, and efflux (404, 567, 1132). One of the most important factors contributing to resistance of *S. marcescens* to β-lactam antibiotics is the inducible overproduction of

cephalosporinase (414). The production of the Ambler Class C β-lactamase (AmpC cephalosporinase) (876) encoded by the chromosomal *ampC* gene (773) in *S. marcescens* has inducible expression conferring resistant to many narrow-spectrum cephalosporins (1022). Moreover overproduction of AmpC (414) as well as variants of the enzyme (652, 775, 1180) were both linked to resistance of *S. marcescens* to expanded-spectrum cephalosporins including cefepime, ceftazidime, and cefpirome.

Acquisition of the Class B metallo-β-lactamases also confers resistance to expanded-spectrum cephalosporins (1177). The first plasmid-mediated carbapenemase was reported in *P. aeruginosa* (1127) and characterised as a class B metallo-β-lactamase (131). Ito *et al.* (482) detected the bla_{IMP-1} gene coding the metallo-β-lactamase in *S. marcescens* in Japan. They noted that strains which acquired the plasmid mediated bla_{IMP} gene were highly resistant to imipenem and were involved in nosocomial infections. Recently, Zhao *et al.* (1196) analyzed 19 imipenem-resistant *S. marcescens* clinical isolates. Six of these isolates were shown to contain the bla_{IMP} and bla_{TEM} genes on a single plasmid, which were expressed constitutively. The investigation concluded that the IMP- and TEM-type β-lactamases play a critical role in the resistance of clinical isolates of *S. marcescens* to ampicillin, cefoxitin, cefotaxime, a limited role to imipenem, but are not responsible for the resistance to aztreonam.

Other mechanisms of resistance to β-lactams have also been identified in *S. marcescens*, including decreased membrane permeability as well as question of Amber Class A β-lactamases and other β-lactamases. Palomar *et al.* (786) investigated the effect of the O-side chain on the permeability of the *S. marcescens* outer membrane to β-lactam antibiotics. They found that O-side chain-defective spontaneous mutants of *S. marcescens* had lower MICs for various β-lactam antibiotics compared with the wild type, and that the outer membrane permeability of the organism to these agents depended on the O-antigen. Naas *et al.*(742) characterised the bla_{Sme-1} gene of the carbapenem resistance *S. marcescens* S6, which conferred resistance to carbapenems, penicillins, aztreonam, cefamandole, and cephalothin. Sequence alignment revealed that the carbapenem-

hydrolyzing Sme-1 is a class A serine β-lactamase with 70% amino acid identity to the pI 6.9 carbapenem-hydrolysing β-lactamase, NMC-A, from *E. cloacae* NOR-1.

3.1.5.1.2 Resistance to aminoglycosides

Resistance to aminoglycosides is acquired by bacteria by preventing the drug from reaching its target site in the ribosome in one of two ways: firstly, alteration in the cell envelope which renders the cell impermeable to the drug, and, secondly the drug itself can be modified by inactivating enzymes which adenylate, acetylate, or phosphorylate the aminoglycoside hydroxyl and amino groups (991). Drug modification is thought to be the more important and most effective, as alteration in the cell envelope usually confers only low level resistance to these agents.

The netilmicin-sensitive, gentamicin-resistant pattern of resistance seen in *S. marcescens* is probably due to the expression of the acetyltransferase AAC(3)-I, an enzyme that inactivates gentamicin but not tobramycin, netilmicin, or amikacin (829). The chromosomally-encoded 6′-*N*-acetyltransferase (AAC(6′)-Ic) of *S. marcescens* which shows activity against amikacin, kanamycin, netilmicin and tobramycin (146), is also involved in aminoglycosides resistance. Although sensitive strains of *S. marcescens* exhibit the naturally-occurring phenotype of a low level expression of AAC(6′)-Ic (407), increased production of the enzyme leads to the development of resistance. Garcia *et al.* (326) found that 90% of their *S. marcescens* isolates were gentamicin-resistant. Dot blot hybridization revealed that the *aac(6')-Ic* gene was present in all the *S. marcescens* isolates examined, and that it was the only amikacin resistance marker in 72% of these isolates. Moreover, the *aac(6')-Ia* and *aac(6')-Ib* genes encoding AAC(6') activity were also found in a number of these isolates. Most of the *S. marcescens* isolates examined (70%), had a triple combination of enzymes AAC(3)-V, AAC(6')-I and APH(3')-I which caused resistance to gentamicin, amikacin, netilmicin and tobramycin.

3.1.5.1.3 Resistance to quinolones

Quinolones are effective antimicrobial agents against *S. marcescens*, and 90% of *S. marcescens* isolates were reported to be inhibited by 0.13 μg/ml of ciprofloxacin (282). However, *S. marcescens* has been found to be resistant to a number of quinolones (555), a resistance that was reported shortly after the initial clinical use of these antibiotics (319). Masecar and Robillard (664) reported that quinolone resistance in a spontaneous *S. marcescens* mutant was due to mutation in the DNA gyrase (*gyrA*) gene. Kim *et al.* (544) characterised the *gyrA* gene of *S. marcescens* ATCC 14756 and determined the mutations in the *gyrA* genes of several quinolone-resistant clinical isolates. They concluded that a mutational alteration in *gyrA* is a common mechanism of quinolone resistance in *S. marcescens.*

Another important mechanism of resistance to quinolones is the decreased accumulation of the antibiotics inside the cell, as mediated by efflux pumps. Kumar and Worobec (567) reported a proton gradient-dependent efflux of fluoroquinolones as a resistance mechanism in *S. marcescens*, and identified a putative RND-pump encoding gene (*sdeB*). In a later study, Kumar *et al.* (566) identified another RND-pump encoding gene *sdeD*, in three *S. marcescens* isolates and characterised the SdeAB RND family multidrug efflux pump. SdeAB was shown to pump out a diverse range of substrates that include fluoroquinolones, chloramphenicol, detergent, ethidium bromide, and organic solvents, and was over expressed in *S. marcescens* strains that are multidrug resistant.

3.1.5.2 *Serratia* and biocides

3.1.5.2.1 Biocide resistance in *Serratia*

Gram-negative bacteria resistance to biocides has been reported for many years. For instance, members of genera *Pseudomonas*, *Proteus* and *Serratia* were all shown to have relatively high resistance to tenside-based disinfectant, and could develop higher resistance if exposed to sub-inhibitory concentrations of these disinfectants (676). In addition, infections caused by biocide resistant organism such as *S. marcescens* and *B.*

cepacia found in solutions of QACs and chlorhexidine have also been reported (796). However, very few studies have looked at the molecular basis of biocide resistance and susceptibility in these organisms including in the genus *Serratia*. One problem is determining whether a strain is resistant or sensitive to a particular antiseptic or disinfectant. Resistance to disinfectants is often used as a relative term in a scientific context. Russell (911) stated that microorganisms are said to be resistant if they are "not killed...by a disinfectant at a concentration used in practice, or if they are not killed by a concentration that kills the majority of cells in a culture or when a strain is not killed by an agent that kills similar strains at a specified concentration".

Nosocomial outbreaks due to *Serratia* strains resistance to biocides, contamination and prolong survival in disinfectant and antiseptics have been reported. One common source of *Serratia* contamination is hand-washes and liquid soaps in medical environments such as hospitals. Sartor *et al.* (945) reported the extrinsic contamination of hospital soap bottles with *S. marcescens* which acted as a continuous source of the organism leading to many outbreaks of infections. They concluded that although the soap used, of which the active ingredients were triethanolamine laurysulfate and betaïne, was marketed as bacteriostatic, it did support the growth of *S. marcescens*, and the application of strict disinfection measures were necessary to stop the *S. marcescens* outbreaks (945).

S. marcescens resistance to biocides has been shown as early as 1951, when Chaplin (150) studied bacterial resistance to QAC disinfectants. He reported that great QAC disinfectant resistance was readily acquired by *S. marcescens* and that apparently there was no limit to the finally tolerated concentration of the disinfectant. He was able to adapt a *S. marcescens* strain to tolerate a concentration of the compound alkyldimethylbenzyl ammonium chloride 1000 times its normal MIC for the bacterium. Chaplin (150) also noticed biochemical changes in the tolerant strain including decrease in electrophoretic mobility and that resistance was removed by treatment with lipase. He concluded that the acquired resistance to the QAC disinfectant was dependent upon the increase lipid content in the tolerant cells.

S. marcescens contaminations of antiseptic and disinfectant solutions with QACs as active agents have been reported (949). Nakashima *et al.* (746) investigated outbreaks of the rare septic arthritis due to *S. marcescens* in an office practice. They determined that patients developed *S. marcescens* septic arthritis following joint injections where a contaminated canister of soaked cotton balls of the compound "Zephiran" was used for antisepsis and disinfection. Zephiran is an aqueous stock solution of the QAC benzalkonium chloride and was used for antisepsis and disinfection. Cotton amongst other materials have been shown to inactivate aqueous QACs after prolong contact (570), which may explain the survival of the epidemic *S. marcescens* strain. Investigators also concluded that contaminated multiple-dose vials of the steroid medication, methylprednisolone also known as "Depo-Medrol", used in the joint injections might have played a key role in the *S. marcescens* septic arthritis outbreaks. The preservative in the latter product is γ-myristyl picolinium chloride, an aqueous QAC.

In a follow up study, Nakashima *et al.* (746) investigated the epidemic strain of *S. marcescens* further by comparing its growth characteristics and susceptibility to QACs with control strains obtained from unrelated nosocomial outbreaks. It was reported that the epidemic strain was able to tolerate 1:100 dilutions of benzalkonium chloride after 72 h, and to grow in multiple-dose vials of methylprednisolone. Control strains on the other hand could not be recovered after 24 h in the same solutions. Investigators concluded that the epidemic strain of *S. marcescens* causing the septic arthritis outbreaks was resistant to low concentrations of the QAC benzalkonium chloride, and speculated that the strain was also cross-resistant to γ-myristyl picolinium chloride, a compound chemically related to benzalkonium chloride.

Chlorhexidine and related biocides solutions contaminated by *Serratia* species have also been reported. Stickler and Thomas (1019) studied the sensitivity of Gram-negative organisms causing urinary tract infections to a number of antiseptics and disinfectants. They isolated *S. marcescens* with a chlorhexidine MIC value of 800 μg/ml, well above the level of 10-15 μg/ml originally reported to inhibit the growth of Gram-negative bacteria (219). In another study, Vigeant et *al.* (1092) reported an outbreak of nosocomial

infections in a university tertiary-care hospital due to a *S. marcescens* contaminated alcohol-free chlorhexidine solutions. The contamination was suspected to be a result of change in the formulation of the chlorhexidine solution from a 4% solution with added isopropyl alcohol to one without alcohol. The solution was further diluted before use. They isolated *S. marcescens* from 84% of the chlorhexidine containers used in the hospital including from the used 4% undiluted solutions. They attributed 80% of *S. marcescens* patients' infections and colonization directly to the contaminated chlorhexidine solutions. Removing the contaminated chlorhexidine and reinstating the original alcohol containing formula resulted in cessation of the epidemic. Vigeant et *al.* (1092) concluded that chlorhexidine without alcohol should not be used as an antiseptic.

A number of studies have reported that various contact lens solutions, especially formulations with chlorhexidine digluconate, are susceptible to contamination by *S. marcescens* (4, 241, 668, 795). Gandhi *et al.* (323) investigated growth and physiological changes that allow *S. marcescens* to survive in contact lens disinfectant solutions containing chlorhexidine gluconate. The latter is commonly used at concentrations of 0.05% in formulations for skin disinfectants. At this concentration, chlorhexidine digluconate has been reported to reduce the population of *S. marcescens* by greater than 99.999% within 10 min (230). Contact lens solutions however, are usually formulated with only 0.005 to 0.006% concentrations of the chlorhexidine gluconate salt. Gandhi *et al.* (323) reported that the *S. marcescens* isolates they studied were able to grow and persist in certain chlorhexidine-based disinfecting solutions recommended for rigid gas-permeable contact lenses. They postulated that *S. marcescens* adapts to chlorhexidine whereby initial exposure to the agent only damages the cells which then recover and adapt. These chlorhexidine-adapted cells had an MIC for chlorhexidine in saline 8-fold higher than non-adapted cells, and exhibited changes in the proteins of their outer membrane and increased adherence to polyethylene. Interestingly the chlorhexidine-adapted cells also persisted and grew in other contact lens solutions with different antimicrobial agents, including polyquaternium-1 and benzalkonium chloride (323).

S. marcescens prolonged survival in chlorhexidine solutions has been demonstrated by Marrie and Costerton (662). They investigated numerous *S. marcescens* infections in a hospital, and found that contaminated chlorhexidine solutions were the source of the outbreaks. The organism was found to survive in chlorhexidine for over 27 months, and isolates from the contaminated solutions were morphologically abnormal showing cell wall disruption or cytoplasmic changes. *S. marcescens* clinical isolates were reported to have a chlorhexidine MIC value of 90 μg/ml, whereas strains from the contaminated stock solutions had an MIC value of 1,024 μg/ml and were able to survive in concentrations up to 20,000 μg/ml (662). In another study, Okuda *et al.* (779) reported that during a prolonged *S. marcescens* outbreak of nosocomial urinary tract infections, 118 of their 131 *S. marcescens* isolated were highly resistant to chlorhexidine with MIC that inhibited the growth of 90% of isolates ranging from 100 to 400 μg/ml. In addition to resistance to chlorhexidine, the strains were resistant to number of antibiotics including sulbenicillin, cefmetazole, gentamicin, and amikacin.

Lannigan and Bryan (582) investigated the mechanism of decreased susceptibility of *S. marcescens* to chlorhexidine. They used the chlorhexidine gluconate resistant *S. marcescens* strain 100 (1018) with a chlorhexidine MIC rage of 800-1000 μg/ml much higher than that of *S. marcescens* 303 or *E. coli* 400 which had MIC ranges of 10-20 μg/ml and 1-25 μg/ml respectively (582). Based on the observation that chlorhexidine caused release of potassium ions from yeast cells (264), Lannigan and Bryan (582) investigated the release of these ions from the resistant *S. marcescens* 100 when exposed to specific concentrations of chlorhexidine. The results showed that whereas *S. marcescens* 303 and *E. coli* 400 showed significant efflux of potassium when exposed to 6 μg/ml chlorhexidine, the resistant strain, *S. marcescens* 100, did not. Spheroplast preparations of *Serratia* isolates were also tested for potassium efflux, and revealed that the presence of the outer membrane had little effect on potassium release. It was concluded that the mechanism of increased resistance of *S. marcescens* 100 to chlorhexidine was a change in the inner membrane (582).

Contamination of triclosan formulations with *Serratia* has also been reported (49). McNaughton *et al.* (687) specifically described a case of *Serratia* infection due to intrinsically contaminated triclosan in infants in a newborn nursery. *S. marcescens* was isolated from the 0.5% triclosan soap that was used for admission baths and unopened bottles of the biocide. Although the effectiveness of 1.5% triclosan soap in eliminating *S. marcescens* has been reported, especially following repeated use (50), in the case of the 0.5% triclosan soap, the organism was clearly able to survive. As a result of the investigation, the soap was withdrawn from the hospital and replaced with non-medicated single-use bar soap, and no further cases of infection with *S. marcescens* were identified (687).

Reports of *Serratia* contamination of other biocide solutions included that by Bosi *et al.* (88) who described *S. marcescens* nosocomial outbreaks in 16 patients attributed to contaminated hexetidine antiseptic solution. Stephen and Lalitha (1015), investigated 28 *S. marcescens* strains during a two-month outbreak of infection in 17 obstetric patients and 11 newborns. The source of infection was attributed to a contaminated batch of cream containing cetrimide that was used during pelvic examination. Ehrenkranz et *al.* (258) demonstrated *S. marcescens* contamination of the QAC disinfectant dimethyl benzyl ammonium chloride, which was sprayed before operations in a heart surgery room. Infections by the contaminating organism complicated the cardiopulmonary bypass operations and were shown to be able to grow in two to four formulations of the QAC disinfectant. Langsrud *et al.* (581) reported the isolation of strains of *S. marcescens* from disinfecting footbaths containing TEGO 103G (amphoteric disinfectant) or TP-99 (alkyl amino acetate-based disinfectant) in five of six dairy factories. These strains were not killed by the recommended in use concentrations of the disinfectants TEGO 103G, TEGO 51 or benzalkonium chloride, but were eliminated with disinfectants based on peracetic acid, hypochlorite, QACs, and alkyl amino acetate. The ability of *S. marcescens* to survive and multiply in the in-use concentrations of disinfectants was attributed to disinfectant resistance.

3.1.5.2.2 Molecular basis of biocide resistance and susceptibility in *Serratia*

One of the few studies which looked at the molecular basis of biocide resistance or susceptibility in *Serratia*, was that undertaken by Codling *et al.* (179). The authors used random transposon mutagenesis to investigate the genes involved in *Serratia* resistance to the QAC biocide, polyquaternium-1, used commercially in a contact lens disinfecting solution. Using the mini-Tn*5*Km2 transposon system, 92 *S. marcescens* mutants with increased susceptibility to polyquaternium-1 were isolated, because they failed to grow at sub-lethal concentration of the biocide. The genes disrupted by the transposon insertions were identified in 19 of the randomly chosen polyquaternium-1 susceptible mutants (Table 3.2). These genes fell into five major functional classes: membrane-associated (including efflux pumps and permeases), biosynthesis and metabolism, gene regulation, virulence, and unknown function (179). The fact that several genes associated with cell membranes were disrupted in the susceptible mutants was not surprising as the QAC biocide has already been shown to damage the cytoplasmic membrane of *S. marcescens* (180). Another interesting result of the study was that over 75% of the *S. marcescens* polyquaternium-1 susceptible mutants exhibited decrease susceptibility to at least one antibiotic tested (Table 3.2). However, 36% of the susceptible mutants also showed an increased resistance to at least one of the following antibiotics; ciprofloxacin, ticarcillin, meropenem or trimethoprim (Table 3.2). These results suggested that antimicrobial resistance mechanisms in bacteria are often linked at the molecular level, and disruption of one of these mechanisms may influence not only biocide susceptibility but also antibiotic resistance patterns.

The molecular basis of *Serratia* resistance to formaldehyde has also been investigated. Resistance to this disinfectant has been reported in many bacteria especially in Gram-negatives such as members of the family *Enterobacteriaceae* and *Pseudomonas* species (523, 941, 1157). Kaulfers and Brandt (523) isolated a conjugative plasmid determining formaldehyde resistance in *E. coli*. This plasmid-mediated resistance was also demonstrated in a clinical strain of *S. marcescens* that was 4-6 times more resistant to formaldehyde than other strains. Conjugation, transformation and plasmid curing

experiments demonstrated that the formaldehyde resistance in the *S. marcescens* strain was plasmid mediated and transferable to *E. coli* (525). This resistance was further investigated by Kaulfers *et al.* (524) where the authors examined the outer membrane of *E. coli* and *S. marcescens* strains both lacking and containing the resistance plasmid. They noticed that although there was no significant immunological difference in the lipopolysaccharide of the strains, sensitive variants had higher protein content in their outer membrane than the resistant strains, and had less surface hydrophobicity. Further investigations into the plasmid mediated formaldehyde resistance in *E. coli* VU3695 revealed that cell extracts of latter and other formaldehyde-resistant strains of *S. marcescens*, *Citrobacter freundii*, *E. cloacae* and *K. pneumoniae*, all showed formaldehyde dehydrogenase activities (941).

A later study by Kummerle et *al* (569) confirmed that the formaldehyde resistance mechanism in the formaldehyde-resistant *E. coli* VU3695 was based on the enzymatic degradation of formaldehyde by a formaldehyde dehydrogenase. They sequenced a large 4.6 Kb plasmid DNA fragment containing the formaldehyde resistance gene from the *E. coli* VU3695 strain, and demonstrated that it contained a single open reading frame (ORF) encoding a glutathione-dependent formaldehyde dehydrogenase (569). The latter is a member of the superfamily of zinc-containing alcohol dehydrogenases found in animal, plant, yeast, and bacterial cells (1081). In *E. coli* VU3695 clinical isolate, the gene conferring formaldehyde resistance was identified as the *adhC* gene located within the large self-transmissible plasmid pVU3695 (569). Further work suggested that *E. coli* VU3695 contains 2 copies of the *adhC* gene, a chromosomal and a plasmid copy, which are actively expressed, with the latter being involved in resistance to exogenous formaldehyde (246). Furthermore, hybridization studies showed that formaldehyde resistant isolates of *S. marcescens*, *E. cloacae*, *C. freundii*, and *K. pneumoniae* harbor a gene highly related to that cloned from pVU3695 (526). Wollmann and Kaulfers (1157) used a large 4.1 Kb DNA fragment of the pVU3695 plasmid which contained the formaldehyde resistance gene as a probe against DNA from formaldehyde-resistant strains of *S. marcescens*, *E. cloacae*, *C. freundii*, and *K. pneumoniae* . DNA hybridization studies showed that all of the above strains hybridized with the DNA probe, indicating

that the resistance gene in *E. coli* pVU3695 and other formaldehyde-resistant *Enterobacteriaceae* have high degree of homology.

Table 3.2. Disrupted genes in the polyquaternium-1 susceptible *S. marcescens* transposon mutants. Adapted and modified from Codling *et al.* (179).

Mutant	Disrupted gene	Decreased MICs	Increased MICs
SM2	Hypothetical unknown	PIP, TMP	
SM3	UDP-galactose-4-epimerase	PIP	
SM4	Hypothetical unknown	AMP, PIP	
SM5	Transcriptional regulator	PIP	
SM6	Membrane protein		CIP
SM8	Hypothetical unknown		TIC
SM10	Sugar binding or transport	AMP, PIP, TMP	
SM12	ATP-binding component of amino acid transport system	AMP, CHL, PIP	
SM14	Microcin H47 secretion protein		CIP
SM15	Transcriptional regulator		MEM
SM16	Hypothetical unknown	PIP	
SM18	Hypothetical unknown	AMP, CHL, PIP,	TMP
SM19	Serralysin (metalloprotease)	AMP, CHL, PIP	
SM20	ArgD (aminotransferase)	AMP	
SM22	Periplasmic murein peptide-binding protein	AMP, CHL, PIP,	CIP
SM24	Arylsulphatase	AMP, CHL	
SM25	Long chain fatty acid CoA ligase	AMP, CHL, MEM, PIP	
SM27	Transporter transmembrane protein	AMP, CHL, PIP,	CIP
SM28	Inner membrane protein, tolerance to colicin E2	TMP	

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; MEM, meropenem; PIP, piperacillin; TIC, ticarcillin; TMP, trimethoprim.

3.2 AIMS

The main aim of this study was to determine the molecular basis of biocide susceptibility and resistance in *S. marcescens*. This was to be achieved by:

a) Examining the susceptibility of the model *S. marcescens* strain Db11 to biocides (*ortho*-phthalaldehyde, triclosan, cetylpyridinium chloride, and chlorhexidine diacetate) and antibiotics.

b) Using transposon mutagenesis to generate random *S. marcescens* Db11 mutants and subsequently screening these mutants to isolate biocide resistant/sensitive derivatives.

c) Evaluating the level of susceptibility of the isolated mutants to the four biocides described above using agar and broth MICs and lethality tests.

d) Using the DNA sequence flanking each transposon in the mutant to identify the disrupted gene by correlation to the *S. marcescens* Db11 genome sequence, and confirming the result by complementation.

e) Comparing the antibiotic profiles of the wild type *S. marcescens* Db11 with those of the biocide mutants to determine any cross-susceptibility between biocides and antibiotics.

The overall hypothesis was that gene transposon mutagenesis would enable the molecular basis of biocide resistance or susceptibility in *S. marcescens* Db11 to be determined. The complete experimental strategy to be followed is outlined schematically in Figure 3.1.

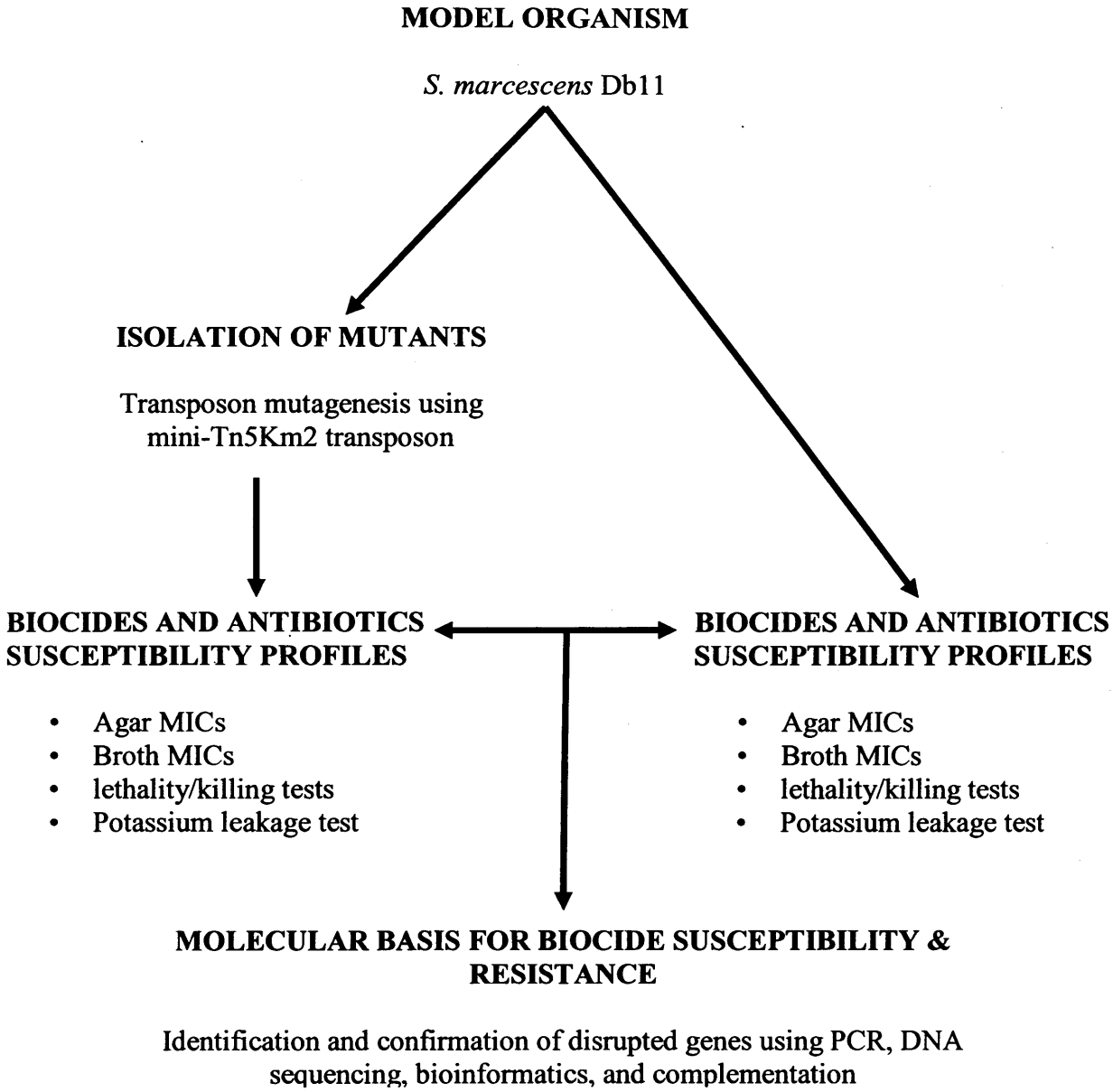


Figure 3.1. Experimental strategy used in the current study.

The experimental strategy used in this study was divided into three major sections: Firstly, biocide and antibiotics susceptibility profiles were to be determined for the model organism *S. marcescens* Db11. This was to be achieved by evaluating agar and broth MICs, the killing effect of biocides, the amount of potassium leaked from biocide-treated cells, and determining the agar MICs for selected antibiotics. Secondly, transposon mutagenesis using mini-Tn5Km2 transposon was to be used to generate and isolate biocide mutants of *S. marcescens* Db11. The biocide susceptibility profiles of these mutants were to be determined as for the wild type *S. marcescens* Db11. Agar MICs of selected antibiotics were also to be determined for the biocide mutants to find out whether change in susceptibility to biocides had an effect on antibiotic sensitivity. Thirdly, the molecular basis of the phenotypic change in the biocide mutants was to be determined by identification of the transposon-disrupted gene in the mutant's genome using molecular techniques including PCR, DNA sequencing and bioinformatics. The results were to be confirmed by complementation analysis.

3.3 MATERIALS AND METHODS

3.3.1 Media, bacteria, and plasmids

3.3.1.1 Strains and plasmids

Bacteria strains and plasmids used in this study are described in Table 3.3.

Table 3.3. Bacterial strains and plasmids.

Strain or plasmid	Reference/catalogue number	Comments
Strains		
Serratia marcescens Db11	(298)	A spontaneous mutant of a non-pigmented *S. marcescens* strain isolated from *Drosophila melanogaster*
Escherichia coli S17.1λpir	Cardiff collection	Containing the delivery vector pUT, carrying mini-Tn*5*Km2 transposon
Escherichia coli Dh5α		Containing pURF047 cloning vector
Escherichia coli OmniMax™2 T1 phage resistant	Invitrogen Corporation, Paisley, UK	Cloning strain used for transformation with the SABE constructs and subsequent transfer to *S. marcescens* Db11 mutants
Escherichia coli	NCTC 1048	Reference strain
Escherichia coli HB101	(290)	Containing mating "helper plasmid" pRK2013, KAN^r, Tra^+, Mob^+, ColE1 replicon
Plasmids		
pUT delivery vector	(223)	Carrying mini-Tn*5*Km2 transposon
pURF047	(1034)	IncW, AMP^r, GEN^r, Mob_, *lacZ*__, Par_, derivative of pURF043
SABE*ompA*	This study	pURF047::*ompA*
SABE*ndpA*	This study	pURF047::*ndpA*

KAN; kanamycin, GEN; gentamicin, AMP; ampicillin, r; resistance

3.3.1.2 Media and growth condition

Both TSB (Difco) and LB broth were used to grow *S. marcescens* cultures and the *E. coli* strains, S17.1λpir, NCTC 10418, and Dh5α. In the case of *E. coli* S17.1λpir the liquid medium was supplemented with 30 μg/ml KAN, and with 10 μg/ml GEN in the case of Dh5α::pURF047 strain. In mating experiments, SOB medium was also used (SOB: 20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl, 2.5 ml 1 M KCl, 10 ml 1 M $MgCl_2$, 900 ml H_2O, pH 7). Growth on solid agar was achieved using TSA (Difco). When required, both solid and liquid media used to grow *S. marcescens* and *E. coli* S17.1λpir were supplemented with the appropriate concentration of antibiotic. Unless mentioned otherwise, all bacteria were grown at 37^0C. Freezer stocks of bacterial strains were prepared by addition of 8% (v/v) DMSO (Fisher Scientific) to a fresh suspension of bacteria. Strains were stored at -80^0C.

3.3.2 Bacterial growth

Growth of bacterial strains was quantified using viable count and by the measurement of optical density. The principles of these methods are outlined in sections 2.3.4 and 2.3.5 respectively. Growth characteristics of bacteria in broth were determined by growth curves showing the change in OD readings at 630 nm of the cultures over time (section 2.3.5). The relationship between OD and viable counts for bacterial strains was also determined and the method was as outlined in section 2.3.6.

3.3.3 Antimicrobial agents

The biocides used in this study were triclosan, chlorhexidine diacetate, cetylpyridinium chloride, and *ortho*-phthalaldehyde. Biocide solutions were prepared fresh on the day as outlined in section 2.3.7. Antibiotics used in this study were kanamycin (KAN), gentamicin (GEN), and polymyxin B (PXB) (Sigma Aldrich, Gillingham, UK). Stock solutions of these antibiotics were prepared in deionised water, filter sterilized, and stored at -20^0C. E-test strips (AB Biodisk, Bio-Stat Ltd) containing amikacin (AMK), azithromycin (AZM), chloramphenicol (CHL), ciprofloxacin (CIP), tobramycin (TOB), trimethoprim/sulfamethoxazole (SXT),

meropenem (MEM), ceftazidime (CAZ), imipenem (IPM), and piperacillin (PIP) were also used.

3.3.4 Biocides MICs determination

Biocides MICs were determined in both liquid and solid media. Biocide solutions were made up fresh on the day of use as described in section 2.3.7, and diluted to the required concentration of use. For MIC determination in liquids, 96-well plates were filled with growth medium containing the appropriate concentration of biocide tested. A culture of the organism to be tested was diluted to an OD of 0.5 ($\approx 10^8$ cfu/ml) and used to inoculate the 96-well plates using a multi-inoculator. The plates were then incubated at 37^0C overnight. Inhibition of growth was determined by reading the OD of cultures at 630 nm using an automated plate reader and comparing the results against controls grown in the absence of the biocide. Broth MICs were taken as the minimal concentrations of biocides that had mean OD values which showed no statistically significant difference from the mean value of the blank. MIC of DMSO for the tested organisms was also determined in broth as described above to evaluate DMSO toxicity on the organisms. Similarly, MICs on agar were determined using TSA plates containing the appropriate concentrations of biocides, inoculated with bacterial cultures of OD_{630nm} of 0.5, and observing inhibition of growth after overnight incubation at 37^0C.

3.3.5 Determination of biocides lethal effects

The lethal effects of triclosan, *ortho*-phthalaldehyde, cetylpyridium chloride and chlorhexidine diacetate on suspensions of S. *marcescens* were determined as follows:

3.3.5.1 Preparation of cell suspensions

S. marcescens cultures were grown overnight at 37^0C. Cells were then washed twice in 0.85% saline after collection by centrifugation at 3000 rpm for 10 minutes, to remove culture media which might interact with the test biocides. The cells were resuspended in 0.85% saline and diluted to an OD_{630nm} of 0.5 ($\approx 6.10^8$ cfu/ml).

3.3.5.2 Controls and neutraliser tests

A) Sterile deionised water control

Sterile deionised water was used as a general control. A 100 µl of the washed cells suspension prepared in section 3.3.5.1, were added to 900 µl of sterile deionised water. The suspension was left for 5 minutes then a viable count was performed on the suspension.

B) The neutraliser effect test

To ensure that the neutraliser used to terminate biocide activity was not toxic to the tested organism, 100 µl of the washed bacterial suspension (section 3.3.5.1) was added to 900 µl of the neutraliser and left for 5 minutes. Viable counts were performed on the suspension and the number of viable cells was compared to that of the sterile deionised water control.

C) The neutraliser efficiency test

To ensure that the neutraliser being used was effective at terminating the activity of the biocides tested, 100 µl of biocide at a lethal concentration to the bacteria tested was added to 800 µl of neutraliser. A 100 µl of the washed cells (section 3.3.5.1) was then added to the mixture and left in contact with the biocide and the neutraliser for 5 minutes. Viable counts were performed on the suspension and the number of viable cells was compared to that of the sterile deionised water control. The following neutralisers were tested for efficiency and effect on cells: 0.75% azolectin in 5% Tween 80, 0.5 % (w/v) sodium bisulphite and 5% (w/v) glycine. The neutraliser that was most efficient at terminating the activity of the test biocide while having the least effect on the test cells was used for further suspension test experiments.

3.3.5.3 Suspension (killing) tests

The lethal effects of triclosan, *ortho*-phthalaldehyde, cetylpyridium chloride and chlorhexidine diacetate on suspensions of *S. marcescens* Db11 were determined by transferring 100 µl of the washed cell suspension (section 3.3.5.1) into 900 µl of biocide. The cells were left in contact with the biocide for a set period of time (5, 10, 20 and 30 min) at room temperature. After the contact period ended a 100 µl of the

suspension (cells and biocide) was transferred into 900 µl of the appropriate neutraliser to terminate the activity of the test biocide. Triclosan, cetylpyridium chloride, and chlorhexidine diacetate were neutralised in 0.75% azolectin in 5% Tween 80, while *ortho*-phthalaldehyde was neutralised in 5% (w/v) glycine. The suspension was left for a further 5 min then the viable cells enumerated on TSA. The number of viable cells was compared to a control whereby the biocide was replaced with sterile deionised water. The biocide lethal effect on the cells was expressed as a $\log_{10}$ reduction in cell number as follows:

$\log_{10}$ reduction in cell number = Log N_c –Log N_t

where N_c and N_t represent the number of cfu/ml in the control and the biocide test solutions.

3.3.5.4 DMSO toxicity test

As both triclosan and *ortho*-phthalaldehyde were dissolved in DMSO, DMSO toxicity to the cells was also determined. The maximum concentration of DMSO used in this study was 2%. A 100 µl of cells suspension prepared in section 3.3.5.1 was put in contact with 900 µl of a 6% DMSO solution, and a viable count on the cells was performed after 5, 10, 20 and 30 min exposure. The $\log_{10}$ reduction in number of cfu/ml in the cell suspensions was calculated and compared to a control to determine the effect of DMSO on cells.

3.3.6 Determination of potassium leakage

The amount of potassium leakage from bacterial cells exposed to biocides was measured using an atomic absorption spectrophotometer. The method was adapted from that used by Suller and Russell for measuring triclosan-induced cytoplasmic membrane damage via potassium leakage (1031). Briefly, cultures were grown on the surface of TSA plates at 37^0C for 24 h. Cells were then emulsified in 5 ml of 0.9% NaCl (Sigma Aldrich, Gillingham, UK), washed three times by centrifugation and resuspended in the appropriate volume of 0.9% NaCl to give a bacterial concentration of $\approx 6.10^8$ cfu/ml. The cells were then exposed to the appropriate concentration of biocide for a specific period of time. The suspensions were then filtered through a 0.2 µm cellulose nitrate filter (Sartorius, Gottingen, Germany) to remove cellular

material. The potassium concentration in the supernatant was then measured using an atomic absorption spectrophotometer. As a negative control, cells were incubated in biocide-free NaCl solution, and the potassium concentration in the supernatant was measured.

3.3.7 Antibiotic MICs determination

Antibiotic MICs for bacteria were determined using E-test on TSA agar according to the manufacturer's instructions (AB Biodisk, Bio-Stat Ltd). Briefly, organisms to be tested were grown in TSB overnight at 37^0C as described in section 3.3.1.2. When *Serratia* transposon mutants were tested, TSB was supplied with kanamycin to a concentration of 100 μg/ml. Cells were then washed twice by centrifugation at 3000 rpm for 10 min to remove culture media, which might interact with the test antibiotic. An inoculum of approximately 10^8 cfu/ml was then obtained by serial dilution in 0.85% saline. A sterile swab was immersed into the cell suspension, excess culture was removed, and the culture was then spread evenly in three directions across a well dried TSA agar plate. Plates were then left to dry for 15 min at room temperature, prior to the application of the E-test strip onto the agar surface. Plates were then incubated for 24 h at 37^0C and analysed. Determination of the antibiotics MICs by E-test were performed in triplicate whenever possible, and the MICs were interpreted at the point of intersection between the inhibition zone and the E-test strip (Chapter 2, Figure 2.4).

3.3.8 Nucleic acid extraction and manipulation

3.3.8.1 Genomic DNA extraction

Three methods were used for DNA extraction from bacteria: the chelex 100 resin method, the boiling method, and the lysis and salting out method. Principles of the first two methods are outlined in section 2.3.11.1. DNA extraction using the lysis and salting out method was as follows.

Cultures used for isolating genomic DNA were grown overnight in 3 ml of the appropriate broth at 37^0C. The bacterial suspension was centrifuged for 10 min at 3000 rpm and the pellet thoroughly resuspended in 100 μl of TE buffer (10 mM Tris-

Cl pH 8, 10 mM EDTA pH 8). A 100 µl of the resulting suspension was added to 500 µl of lysis buffer (1% SDS, 50 mM Tris-Cl pH8, 50 mM EDTA pH 8) containing 0.5 mg/ml pronase, contained in 2 ml screw cap tube with about 500 µl of 0.1 mm diameter washed glass beads. The tube was then placed in a mechanical beater at (speed setting 25) for 10 sec prior to incubation at 37^0C for 60 min. The tube was then centrifuged at 13000 rpm for 1 min to pull down the formed bubbles and 200 µl of saturated ammonium acetate was added to the lysate and mixed by pulsing on bead-beater for 5 sec at (20 speed). The tube was then centrifuged at 13000 rpm for 1 min to pull down the formed bubbles and 600 µl of chloroform was added to the lysate. The tube was pulsed on bead-beater for 5 sec at (20 speed) to mix then centrifuged at 13000 rpm for 3 min. Following centrifugation the top, clear, aqueous phase of the lysate (≈ 400 µl) was removed to a clean sterile 1.5 ml Hi-Yield micro-tube containing 1 ml 100% ethanol. The tube was inverted several times to mix until an observable DNA cloth was obtained. DNA was collected by centrifugation at 13000 rpm for 5 min, and the pellet washed in 500 µl of 70% ethanol. All the ethanol was removed and the resulting DNA pellet was dried under vacuum for 10 min. DNA was then dissolved in either 300 µl TE (10 mM Tris-Cl pH8, 0.1 mM EDTA), or 300 µl sterile polished water both containing 0.5 µg/ml RNase (Sigma-Aldrich Ltd, UK) and incubated at 37^0C for 1 to 2 h. DNA was stored at 4^0C or deep-frozen at -20^0C prior to further use.

3.3.8.2 Plasmid DNA preparation

Plasmid DNA was extracted from bacterial cultures using The Wizard® plus DNA purification system. The Wizard® plus SV mini-prep DNA purification system was used according to the manufacturer's instruction (Promega Biosciences Inc, Couthampton, UK) to prepare plasmid DNA from 5 ml bacterial cultures. Plasmid DNA extraction using the standard alkaline lysis method, which principles are outlined in section 2.3.11.2, was also used

3.3.8.3 RNA extraction

RNA was extracted from cells in their mid-log phase of growth as follows. Bacteria were grown in the appropriate growth medium until they reached their mid-log phase

of growth. The suspensions were then diluted to an OD of 0.5 at 630 nm to achieve equal number of cells (≈ 6.10^8 cfu/ml). The cells were then harvested by centrifugation (5000 *g*) for 5 min at 4^0C and the supernatants completely removed. The cells were then snap frozen in liquid nitrogen and left at -80^0C overnight. The following day, the bacterial pellets were loosened by flicking the bottom of the tubes and resuspended in 100 µl of TE buffer containing 1 mg/ml lysozyme. A 350 µl of buffer RLT (Qiagen Ltd, UK) was added to the samples, mixed thoroughly by vortexing and then 250 µl of 100% ethanol was added. The samples were mixed thoroughly by pipetting before being applied to the specialised RNeasy mini columns placed in a 2 ml collection tube (Qiagen Ltd, UK). The samples were then centrifuged at 8000 *g* for 15 sec and the flow-through discarded. A 700 µl of buffer RW1 (Qiagen Ltd, UK) was then added to the samples which were then centrifuged at 8000 *g* for another 15 sec. The RNeasy columns were then transferred into a 2 ml collection tube and 500 µl of buffer RPE (Qiagen Ltd, UK) was added, followed by a centrifugation step at 8000 *g* for 2 min. The RNeasy columns were transferred into new 1.5 ml collection tubes and RNA was eluted by the addition of 30 µl of RNase-free water directly onto the RNeasy silica-gel-membranes of the columns, and centrifugation at 8000 *g* for 1 min. RNA samples were stored at -20^0C for further use.

3.3.9 Whole cell protein extraction and analysis

Whole cell protein was extracted from cells and analysed using protein gel electrophoresis system and reagents (Bio-Rad Laboratories Ltd, Hertfordshire, UK) according to the manufacturer's instructions. Briefly, overnight suspensions of cells were centrifuged at 3000 rpm for 10 min and the pellet resuspended in phosphate buffered saline (PBS) to an OD of 1 at 600 nm. A 1 ml of the resuspension was then transferred into a 1.5 ml micro-tube, centrifuged at 13000 rpm for 2 min, then resuspended in 250 µl PBS. A 100 µl of the resulting suspension was then mixed with 33 µl of 4X XT sample buffer (Bio-Rad Laboratories Ltd, Hertfordshire, UK) and 6.6 µl of 20X XT reducing agent (Bio-Rad Laboratories Ltd, Hertfordshire, UK). Varying dilutions of the cell suspension in PBS were also used as appropriate to determine the dilution that gives the optimum clearly visible protein profile on gel. The mixture was boiled for 5 min in a water bath, centrifuged at 13000 rpm for 3min to removed cellular debris and the supernatant was placed into a clean 1.5 ml micro-tube. The

protein samples were loaded into the gel according to the manufacturer's instructions (Bio-Rad Laboratories Ltd, Hertfordshire, UK) and run in 10X Tris/glycine/SDS running buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS) (Bio-Rad Laboratories Ltd, Hertfordshire, UK) at 80 volts for 1 hr. The gel was then stained with Bio-Safe coomassie strain according to the manufacture's instructions (Bio-Rad Laboratories Ltd, Hertfordshire, UK). A precision Plus dual colour protein standard was used for sizing (Bio-Rad Laboratories Ltd, Hertfordshire, UK).

3.3.10 Polymerase chain reaction (PCR)

PCR primers and target DNA for amplification are listed in Table 3.4. Primers were ordered from MWG Ltd Biotech (Milton Keynes, UK). The primers were dissolved to 100 pmol/µl in accordance with the manufacturer's instructions, then checked on an agarose gel. Novel primers designed in this study were generated using "primer 3" software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) or designed manually. Regions of DNA were amplified using the polymerase chain reaction (933) as described by Sambrook *et al* (936). Unless otherwise stated, PCR amplifications were performed using Promega PCR kit reagents (Promega corporation Inc, Southampton, UK), and in a standard 25 µl reaction mixture containing PCR buffer (1X manufacturer's stock), 1.5 mM $MgCl_2$, 200 µM of each of the deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), 1 unit of Taq polymerase, 5 to 10 pmol of each forward and reverse primers and roughly 10-50 ng/µl of DNA template. Both a positive (template DNA) and negative (sterile polished water) control were included to confirm amplification of the correct DNA sequence. DNA amplification was carried out with a MJ Research PTC-200 Thermal cycler using block control and a heated lid. The standard reaction program consisted of initial 5 to 10 min DNA denaturation at 94^0C as appropriate, followed by 30 cycles of 30 sec to 1 min denaturation at 94^0C, 30 sec to 1 min of primer annealing at the appropriate temperature (Table 3.4), and a 1.5 to 3 min elongation step at 72^0C. The process was terminated by a final elongation stage for 5 min at 72^0C. Specific conditions used for non-standard PCR are stated in the appropriate section.

Randomly amplified polymorphic DNA (RAPD) analysis was performed on the *S. marcescens* Db11 transposon mutants to confirm that they were all the same strain of

Serratia. The analysis was performed using primer 270 (644) (Table 3.4), and results confirmed using primer 272 (644) (Table 3.4). The RAPD PCR reaction mixtures (25 µl) were set up as described above, containing PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each of the deoxynucleotide triphosphates (dNTPs), 1 unit of Taq polymerase, 40 pmol of primer 270, or 272, and roughly 10-50 ng/µl of DNA template. DNA was extracted from bacteria using chelex method (sections 2.3.11.1.1). DNA from the *S. marcescens* Db11, *S. marcescens* Db11 transposon mutants, and *E. coli* S17.1λpir were included in the analysis. DNA amplification was carried out with a MJ Research PTC-200 Thermal cycler using block control and a heated lid and consisted of two steps. Step 1 consisted of initial 5 min DNA denaturation at 94^0C, followed by 4 cycles of 5 min denaturation at 36^0C, 5 min of primer annealing at 72^0C, and a 5 min elongation step at 94^0C. Step two consisted of 30 cycles of initial 1 min DNA denaturation at 94^0C, followed by 1 min denaturation at 36^0C, 1 min of primer annealing at 72^0C, and a 1 min elongation step at 72^0C. The process was terminated by a final elongation stage for 10 min at 72^0C. RAPD products were then separated by electrophoresis (section 2.3.12) in 1.5% agarose gels at 100 volts for 3 h. After the gels were stained and photographed, the RAPD fingerprints were analyzed by eye to determine similarities.

Table 3.4. Details of the PCR primers and DNA targets used in this study.

Primer	Sequence 5'- 3'	Product size (bp)	Annealing temperature	Specificity/ use	Reference /source
NPTII_F NPTII_R	CTTGCTCGAGGCCGCGATTAAATT TTCCATAGGATGGCAAGATCCTGG	676	61^0C 61^0C	Amplify the *nptII* kanamycin resistance gene.	(498)
Primer 270 Primer 272	TGCGCGCGGG AGCGGGCCAA		See section 3.3.10	RAPD analysis	(644)
Primer 1 Primer 2a Primer 2b Primer 2c Primer 3 Primer 4	TTTTTACACTGATGAATGTTCCG GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT CGGATTACAGCCGGATCCCCG GGCCACGCGTCGACTAGTAC		See section 3.3.12	Amplify the mini-Tn*5*Km2 insertion junction in the *S. marcescens* Db11 mutants chromosome	(655)
ompA_F ompA_R	GCTACCCAATTGCTGACGAT AGCCCTGAGGCTGAGTTACA	743	50^0C 50^0C	Amplify within the *ompA* gene.	This study
ompA_F_Eco ompA_R_Eco	TATTAT**GAATTC**TTGGCAAAGATCCCAAG * TTATTA**GAATTC**TCGGCTGGTTGCCTGAG *	2417	50^0C 50^0C	Amplify the *ompA* gene with *Eco*RI tails for cloning.	This study
ndpA-F-Bam ndpA-R-Bam	TATA**GGATCC**CGACGACATGCTGAAGGC ** TATA **GGATCC**CACACCACCGGATTGAGG **	2739	58^0C 58^0C	Amplify the *ndpA* gene with *Bam*HI tails for cloning	This study

* *Eco*RI tails are shown in bold and underlined

** *Bam*HI tails are shown in bold

3.3.11 Mutant generation and screening

3.3.11.1 Conjugational transfer of plasmids to *S. marcescens*

The method used was modified from Lewenza *et al.* (602). Overnight cultures of both donor and recipient strains, *E. coli* S17.1λpir and *S. marcescens* Db11 respectively, were grown in SOB medium (section 3.4.1.2) at 37^{0}C. The cultures were then centrifuged at 2000 rpm for 5 min and washed with SOB medium. The washing was repeated a second time, then cultures were resuspended in 3 ml of LB broth. In a new sterile tube, 1 ml of each of the donor and recipient bacteria were mixed. 100 µl of the mixed *E. coli* and *S. marcescens* strains were spotted onto sterile 0.2 µm pore-size nitrocellulose filters (Whatman, Miadstone, UK), placed on TSA containing 10 mM $MgSO_4$, and incubated for 5 h at 37^{0}C. The donor and recipient strains were also spotted individually as described above for controls. After incubation, the filters were removed from the plates and the cells resuspended in 1 ml of LB broth. The cells were spread plated (100 µl per plate) onto TSA agar plates containing 100 units/ml PXB (to select for *S. marcescens* and kill *E. coli*) and 100 µg/ml KAN (to select for cells containing mini-Tn*5*Km2). The plates were incubated for 24 h at 37^{0}C and then checked for transconjugants. Viable counts on the *S. marcescens* recipient were carried out as described in section 2.3.4 to determine the frequency of transconjugation.

3.3.11.2 Mutants screening and isolation of biocides sensitive/resistant mutants

Out of the transconjugants that grew on the selective TSA plates after 24 h, 6000 colonies were picked into 96-well plates containing 150 µl LB with 100 µg/ml KAN and incubated overnight at 37^{0}C. The following day the appropriate volume of DMSO to make up 8% of the total culture volume in each well was added and the plates were stored at -80^{0}C. Triclosan, cetylpyridium chloride, chlorhexidine diacetate, and *ortho*-phthalaldehyde sensitive mutants were isolated by replica plating from the 96-well plates onto first master TSA plates containing 100 µg/ml KAN and onto selective TSA plates with 100 µg/ml KAN and containing different screening concentrations of biocides

(Chapter 2, Figure 2.7). These were as follows: triclosan at 2500, 3000, 3500, and 4000 µg/ml, *ortho*-phthalaldehyde at 2400, 2800, 3000, and 3200 µg/ml, cetylpyridium chloride at 50, 70, 90, and 110 µg/ml, chlorhexidine diacetate at 10, 14, 18, and 26 µg/ml. After overnight incubation at 37^0C, those mutants that grew on the master TSA/KAN plates but not on the selective, biocides containing plates, were picked, re-plated, and stored in TSB containing 8% DMSO at -80^0C. Colonies that grew at higher biocide concentrations were also isolated for probable resistance. These mutants were further characterised along with two random mutants which showed no changes in sensitivity to any of the biocides and which were used as controls.

3.3.12 Identification of disrupted genes

The site of insertion of the mini-Tn*5*Km2 transposon into the biocide mutants was determined by sequencing the transposon-chromosome insertion junction. This was achieved by a two stage PCR which was adapted from a previously described method (655), which uses a two modified primers 1 and 3 (Table 3.4) which were re-designed (498) to target the primers to mini-Tn*5*Km2. The method also included alterations to accommodate the use of three second primers (2a, 2b, and 2c) (Table 3.4) simultaneously in a multiplex PCR reaction to increase the chance of amplifying the target gene sequence. Figures 3.2 and 3.3 show a schematic of the principles of the two stage PCR reaction and the reaction conditions respectively.

The resulting PCR products were purified using the QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen Ltd, UK). The products were then sequenced using sequencing reactions using 1.6 pmol of primer 3 with Applied Biosystems Big Dye Terminator ready reaction mix version 3.1 and analysed using an ABI-PRISM 3100 Genetic Analyser capillary electrophoresis system running Performance Optimised Polymer 6 (POP-6) according to the manufacture's instructions (Applied Biosystems, Foster City, CA, USA). The transposon sequence was then removed and the chromosome sequence at the point of insertion was obtained. This

sequence was then used along with Artemis software (929) to pinpoint the exact location of insertion of the transposon into the *S. marcescens* Db11 genome (Appendix I).

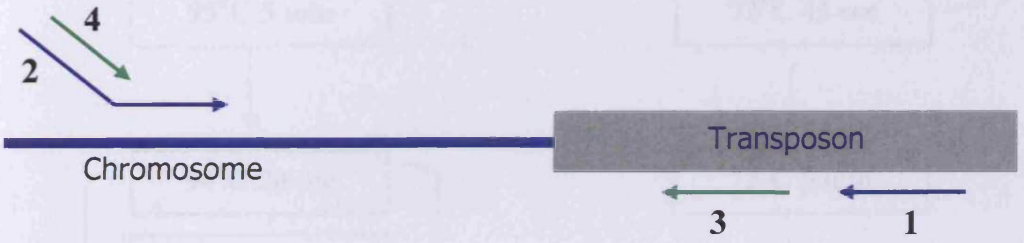


Figure 3.2. PCR amplification of the mini-Tn5Km2 transposon insertion junction.

In the first PCR reaction, chromosomal DNA is amplified using primer 1 (transposon specific) and primer 2 (degenerate mixture designed to hybridize at any site in genome, with a unique 5' end sequence). In the second PCR reaction, theproduct of first PCR is amplified using primer 3 (transposon specific) and primer 4 (hybridizes to the unique sequence introduced at the end of primer 2). The result is the amplification of the transposon-chromosome junction

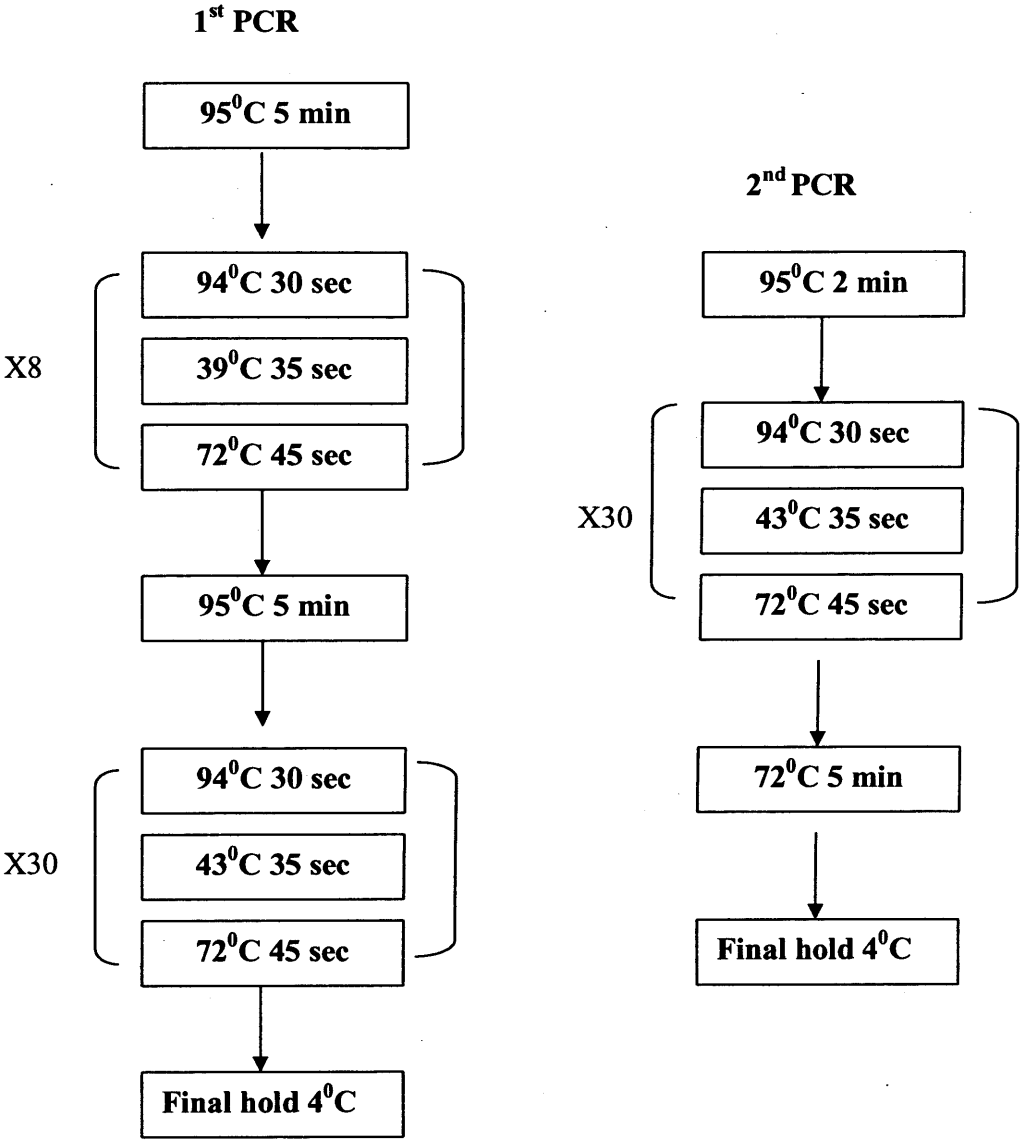


Figure 3.3. Two step PCR reaction used to amplify and sequence the mini-Tn*5*Km2 transposon insertion in *S. marcescens* Db11 genome (655).

PCR protocol for the first and second PCR reactions (Figure 3.2) used to amplify the mini-Tn*5*Km2 transposon-chromosome junction in the *S. marcescens* Db11 mutants.

3.3.13 Complementation of mutants

Biocide mutants with disrupted genes identified were complemented as follows:

3.3.13.1 Cloning vector extraction and primer design for mutated genes

Plasmid pURF047 was chosen to be used as a vector for complementation in *S. marcescens*. The plasmid was extracted from *E. coli* DH5α using The Wizard® plus SV mini-prep DNA purification system as described in section 3.3.8.2. The wild type sequences of transposon-mutated genes to be complemented were analysed using DSGene (Accelrys Softwar Inc, Cambridge, UK) to identify the position of restriction sites. Restriction endonucleases cutting sites that fell inside the disrupted target genes were not used in the cloning steps as it is necessary to keep the target genes intact. The primers were designed to introduce *EcoR*I, or *Bam*HI restriction sites, and 6 bp tails containing only adenine and thiamine to assist effective endonuclease reactions.

3.3.13.2 Epicentre "Failsafe" PCR amplification of disrupted genes for complementation

To ensure error-free amplification of DNA, the epicentre "Failsafe" PCR PriMix selection kit was used to amplify the disrupted genes at optimal levels of $MgCl_2$ and FailSafe™ PCR Enhancer with Betaine. The system includes the FailSafe™ PCR enzyme mix, a blend of thermostable enzymes with at least 3 times the nucleotide incorporation accuracy of the *Taq* DNA polymerase. Along with the FailSafe™ PCR enzyme mix, the system includes a set of 12 FailSafe™ PCR PriMixes that cover a matrix of enzyme-specific PCR conditions that are optimal for amplifying different sequences.

PCR reactions were carried out in a 50 µl reaction mixtures using a MJ Research PTC-200 Thermal cycler as described in section 3.3.10. The PCR reactions consisted of an initial denaturation at 94^0C for 2 min, followed by 30 cycles of 1 min denaturation at 94^0C, 1 min of primer annealing at the appropriate temperature (Table 3.4), and a 2 min

elongation step at 72^{0}C. The process was terminated by a final elongation stage for 5 min at 72^{0}C. PCR products were visualized by agarose gel electrophoresis (section 2.3.12). The Failsafe PCR reaction that gave the most concentrated and discrete electrophoresis band of the correct size of each gene, was purified into 30 µl of sterile polished water, using the QIAquick PCR purification system according to the manufacturer's instructions (Qiagen Ltd, Crawley, UK).

3.3.13.3 Ligation of the amplified DNA fragments into pURF047 vector

Restriction endonuclease digests using *EcoR*I, or *Bam*HI as appropriate were used to cut both the pURF047 vector and the Failsafe PCR reaction products. The digest was performed according to the manufacturer's instructions (Promega Corporation Inc, Southampton, UK) in 50 µl reaction mixture for the vector pURF047 and 20 µl reaction for the Failsafe PCR fragments. The digests were incubated at 37^{0}C for 5 h then heat inactivated at 65^{0}C for 15 min and checked using agarose gel electrophoresis (section 2.3.12). DNA fragments were ligated in 20 µl reaction mixtures using a ligation kit (Promega Corporation Inc, Southampton, UK) and according to the manufacturer's instructions. The reaction mixtures contained 2 µl of 10X ligation buffer (10% v/v), 1 µl T4 DNA ligase, 4 µl vector DNA, and 8 µl of substrate DNA. A vector control reaction where the substrate DNA was replaced with sterile polished water was also included. The ligation mixtures were left incubated at 19^{0}C overnight before use for transformation.

3.3.13.4 Transformation of the One Shot OmniMAX^{TM} 2 T1 phage-resistant chemically competent *E. coli*

The One Shot OmniMAX^{TM} 2 T1^R *E. coli* cells (Invitrogen Corporation, Paisley, UK) were thawed on ice, and 2.5 µl of the ligation mix (section 3.3.13.3) was gently mixed with 50 µl aliquot of the competent cells. The cells were incubated on ice for 30 min then heat-shocked at 42^{0}C for 30 sec. The cells were then incubated on ice for a further 2 min before a 250 µl of a pre-warmed SOC medium (Invitrogen) was added. The transformation mix was then incubated with shaking at 225 rpm for 1 h at 37^{0}C before 700 µl of SOC medium was added to the mix to bring the final volume to 1 ml. 100 µl

aliquots of the transformation mix were then spread onto TSA plates containing 10 μg/ml gentamicin to select for the plasmid, and XTRA-Blue Plus X-Gal/IPTG solution (MP Biomedicals, London, UK) at 2.8 μl/ml to enable the growth of individual clones to produce visible colonies. The plates were incubated at 37^0C and the growing colonies were then analyzed.

3.3.13.5 Confirmation of cloned genes by PCR and by DNA fragment excision

White colonies resulting from the transformation experiment (section 3.3.13.4) were isolated and used to generate both genomic DNA and plasmid pURF047 containing inserts. PCR reactions using the appropriate primers for the insert's amplification were carried out using genomic DNA and pURF047 containing inserts from the transformants. Transformants which were positive for the right insert were used for further complementation analysis experiments. In some cases, the successful cloning of the gene was confirmed by extracting the vector from the cells and excising the cloned DNA fragment by digestion with the appropriate restriction enzyme.

3.3.13.6 Tri-parental mating to transfer the cloned gene construct to the host by conjugation

Tri-parental mating was used to introduce the pURF047 construct containing the cloned gene of interest into both the wild type *S. marcescens* Db11 and mutant hosts by conjugation. Bacterial strains were gown on TSA containing the appropriate selection overnight at 37^0C as follows. Donor One Shot OmniMAX^{TM} 2 T1^R *E. coli* transformants (section 3.3.13.4) containing the pURF047 construct (with gene of interest), or donor *E. coli* Dh5α containing pURF047 were grown on TSA containing 10 μg/ml gentamicin. *E. coli* HB101 harboring the "helper plasmid" (pRK2013) was grown on TSA containing 50 μg/ml kanamycin. Recipient cells included wild type *S. marcescens* Db11 grown on TSA or *S. marcescens* mutants grown on TSA containing 100 μg/ml kanamycin. Bacterial strains from plates were suspended in 5 ml TSB with the appropriate selection and grown overnight at 37^0C. Cells were harvested by centrifugation for 10 min at 3000 rpm,

washed twice in TSB containing 10 mM $MgSO_4$, centrifuged and resuspended in 3 ml TSB containing 10 mM $MgSO_4$. At this stage 1 ml of each of the parental strains (donor, recipient and helper) were combined and a 100 µl of the mating mixture was spotted onto sterile 0.2 µm pore-size nitrocellulose filters (Whatman, Maidstone, UK), placed on a dry TSA containing 10 mM $MgSO_4$, and incubated for 5 h at 37^0C. A 100 µl of each of the parental strains were also individually spotted and incubated as above. After incubation, bacteria were resuspended from the filters by vortex-mixing in 1 ml TSB, and serial dilutions of the suspensions were plated onto selective TSA plates. For tri-parental mating with wild type *S. marcescens* Db11 as recipient, the selective plates were TSA containing 10 µg/ml gentamicin and 100 units/ml PXB. For mating involving mutants as recipients, 100 µg/ml kanamycin was added to the above selective plates. After 24 h incubation at 37^0C, colonies from the mating mixture growing on the selective plates were picked and tested for presence of the right construct (section 3.3.13.5).

For every mutant complemented, the pURF047 construct with the cloned gene was introduced not only into the mutant but also into the wild type *S. marcescens* Db11 to determine whether the introduction of a second copy of the gene into the wild type would have any effect on phenotype. Similarly, for each complementation, tri-parental mating was used to introduce the pURF047 plasmid into the wild type *S. marcescens* Db11 and into the mutants using *E. coli* Dh5α as a donor. This was to determine if the introduction of the pURF047 cloning vector on its own had an effect on the phenotype of the wild type or mutants. All complemented mutants and derivatives (wild type containing pURF047, wild type containing pURF047 with cloned gene, and mutant containing pURF047) were stored at -80^0C (section 3.3.1.2).

3.3.13.7 Phenotypic complementation analysis

Transconjugants showing a successful insertion of the pURF047 containing the right insert (section 3.3.13.6), were used in phenotypic complementation analysis. MICs of the biocides were determined for the complemented mutants, and results were compared to those of the wild type *S. marcescens* Db11, *S. marcescens* Db11 containing the pURF047

vector, *S. marcescens* Db11 containing recombinant pURF047 with the right gene insert, the non-complemented mutant, and the mutant containing only pURF047.

3.3.13.8 Reverse transcriptase PCR (RT-PCR)

The reverse transcriptase polymerase chain reaction (RT-PCR) was used to test if target genes were being expressed at the mRNA level. The procedure was performed using Promega RT-PCR kit and according to the manufacturer's instructions (Promega Corporation Inc, Southampton, UK). The RT-PCR reactions used in this study are summarised in Table 3.5. cDNA generated from the RT-PCR reaction was then used to amplify the target gene using the appropriate primers and standard PCR (section 3.3.10).

Table 3.5. Reverse transcriptase PCR.

		Volume added (µl)			
		Experiment	**No-RT[c]**	**Negative control (no-template)**	**Positive control**
Target RNA and Primer combination and denaturation	Nuclease-free H_2O	2	2	4	2
	Random primer	1	1	1	1[a]
	RNA	2	2	-	2[b]
	Total volume[d]	5	5	5	5
		Experiment	**No-RT[c]**	**Negative control**	**Positive control**
Reverse transcription	Nuclease-free H_2O	4.5	5.5	4.5	4.5
	X10 Buffer	4	4	4	4
	$MgCl_2$ (10mM)	4	4	4	4
	dNTP	1	1	1	1
	RNasin Inhibitor	0.5	0.5	0.5	0.5
	ImProm-II Revers trascriptase	1	-	1	1
	Total volume	15	15	15	15

a) 0.5 µg of Oligo$(dT)_{15}$ primers, b) 1 µg of 1.2 Kb kanamycin positive control RNA, c) reactions not containing the reverse transcriptase enzyme, d) volume added to the reverse transcription reaction.

3.4. RESULTS

3.4.1 Growth characteristics of *S. marcescens* Db11

S. marcescens Db11 grew well in both TSB and TSA, and good growth was observed after overnight incubation at 37^0C. On agar, the organism formed smooth mucoid colonies which had entire margins and umbonate elevation. Unlike some other *S. marcescens* strains which are chromogenic and produce the red pigment prodigiosin, strain Db11 is non-pigmented (Figure 3.4). Growth characteristics of *S. marcescens* Db11 in broth was determined by generating growth curves for the organism using OD values (at 630 nm) of *S. marcescens* Db11 cultures read at different times over a period of 15 h (section 3.3.2). As seen from Figure 3.5, growth of *S. marcescens* Db11 followed typical bacterial growth phases. The initial "lag phase" was short extending over 1 h, followed by a "log phase" of growth over approximately 4 h. The "stationary phase" started with a deceleration phase and was the longest phase of growth. The *S. marcescens* Db11 mean specific growth rate (μ) and the culture doubling time (g) were both calculated and had values of 0.9 h^{-1} and 0.77 h respectively. The above investigation was also performed on the control reference strain used in this study (*E. coli* NCTC 1048), and the μ and g values for this strain were 1 h^{-1} and 0.68 h respectively. Statistical analysis demonstrated that the *E. coli* mean specific growth rate was statistically significantly different from that of *S. marcescens* Db11 (Table 3.11).

The relationship between the $OD_{630\ nm}$ values of a *S. marcescens* Db11 culture and its viable count was also investigated (section 3.3.2) to determine the number of cfu present per ml of a standard inoculum used in further investigations. The standard inoculum chosen was a culture with an $OD_{630\ nm}$ value of 0.5. The results showed that the relationship between the OD values and the viable count was linear (Figure 3.6), and that the relationship remained uniform up to an OD value of over 1. A culture with an OD_{630nm} value of 0.5 had approximately 6.10^8 cfu/ml of bacteria (Figure 3.6).

3.4.2 *S. marcescens* Db11 biocide MICs

Susceptibility of *S. marcescens* Db11 to four biocides was investigated by determining the MICs of these biocides for the bacterium (section 3.3.4). The four biocides used were triclosan, cetylpyridinium chloride, chlorhexidine diacetate, and *ortho*-phthalaldehyde. As both triclosan and *ortho*-phthalaldehyde were dissolved in DMSO, toxicity of the latter to *S. marcescens* Db11 was also investigated. The reference *E. coli* NCTC 1048 was also included in the investigations and used for comparative purposes.

MICs for the four biocides were determined in TSA and in TSB, whereas DMSO MICs were determined only in broth (section 3.3.4). Results (Figure 3.7) showed that DMSO only started to have a significant effect on bacterial growth at concentrations over 4% (v/v), much higher than any concentration of this agent used in this study (2%). Hence, it was concluded that DMSO did not have a significant effect on *S. marcescens* Db11 growth in our investigations. The results of biocide MICs (Table 3.6) showed that MIC values were higher in agar than in liquid broth for all biocides. On agar chlorhexidine diacetate was the most effective biocide at inhibiting the growth of *S. marcescens* Db11 with an MIC value of 18 μg/ml, followed by cetylpyridium chloride (MIC = 100 μg/ml). MICs for *ortho*-phthalaldehyde and triclosan were much higher with values of 3000 μg/ml and 3900 μg/ml respectively.

In liquid media on the other hand, cetylpyridium chloride was more effective at inhibiting growth than chlorhexidine diacetate, and these agents had MIC values of 5.5 μg/ml, and 7.5 μg/ml respectively (Figure 3.7). Broth MICs of triclosan and *ortho*-phthalaldehyde were not determined as the interaction between these biocides and the bacterial suspensions resulted in change in the colour of the medium (turning into a milky white colour with increasing concentrations of triclosan and a dark colour, ranging from green to black, depending on concentration, with *ortho*-phthalaldehyde), hence making readings ODs impossible. MIC values for the reference strain *E. coli* NCTC 1048 (Table 3.6), showed that on agar, the latter was more sensitive than *S. marcescens* Db11 for all biocides tested with the exception of *ortho*-phthalaldehyde. Interestingly, the *E. coli* NCTC 1048 MIC value for triclosan on agar was 30000 times lower than that of *S.*

marcescens Db11, while the two organisms had similar MICs for *ortho*-phthalaldehyde. In broth, *E. coli* NCTC 1048 had also MIC values lower than those of *S. marcescens* Db11, for both cetylpyridium chloride and chlorhexidine diacetate. However, the difference in MIC values between the two organisms for these biocides was not as high as that seen on agar (Table 3.6), nevertheless it was statistically significant at the 95% confidence level.

3.4.3 Biocides suspension (killing) tests on *S. marcescens* Db11

The lethal effects of triclosan, *ortho*-phthalaldehyde, cetylpyridium chloride, and chlorhexidine diacetate on *S. marcescens* Db11 was determined using suspension tests (section 3.3.5). The viable counts of a *S. marcescens* Db11 culture subjected to a specific concentration of a biocide was determined at different times of exposure (0, 5, 10, 20, and 30 min). These results were used to calculate the $\log_{10}$ reduction in number of cells caused by the biocide at the corresponding exposure times, hence determining the biocide's lethal effect. The *E. coli* NCTC 1048 reference strain was also included in the investigations and used for comparative purposes. A number of neutralisers were used to neutralise the biocides in the suspension tests. Both the efficiency and effects of these neutralisers on the *S. marcescens* Db11 cells were also investigated (section 3.3.5.2).

3.4.3.1 Neutraliser effect on cells

The effect of neutralisers on *S. marcescens* Db11 cells was investigated (section 3.3.5.2). Three different neutralisers were studied: 0.5 % (w/v) sodium bisulphite, 5% (w/v) glycine and 0.75% azolectin in 5% Tween 80 tested for neutralisation of *ortho*-phthalaldehyde, and 0.75% azolectin in 5% Tween 80 tested for neutralisation of triclosan, cetylpyridium chloride and chlorhexidine diacetate. The results (Table 3.7) showed that both 5% (w/v) glycine and 0.75% azolectin in 5% Tween 80 had no statistically significant effect on the survival of *S. marcescens* cells, whereas 0.5 % (w/v) sodium bisulphite had a lethal effect. The 0.75% azolectin in 5% Tween 80 neutraliser caused only 0.03 $\log_{10}$ reduction in number of cells, whereas 5% (w/v) glycine cause a 10 fold higher (0.30 $\log_{10}$) reduction.

3.4.3.2 Neutraliser efficiency

The efficiency of the three neutralisers at inhibiting the activity of biocides was also investigated. The results (Table 3.8) showed that 0.75% azolectin in 5% Tween 80 was the appropriate neutraliser to use with triclosan, cetylpyridium chloride, and chlorhexidine diacetate, as it was effective at neutralizing all these biocides. Out of the three neutralisers investigated for *ortho*-phthalaldehyde, 5% (w/v) glycine was the best at neutralizing the biocide followed by 0.75% azolectin in 5% Tween 80. The efficiency of 0.5 % (w/v) sodium bisulphite at neutralizing *ortho*-phthalaldehyde was not possible to measure due to the lethal effect this compound had on the cells (Table 3.7). However, a study by Shackelford *et al.* (970) concluded that 0.5 % (w/v) sodium bisulphite was satisfactory at neutralizing the activities of up to 0.5 (w/v) *ortho*-phthalaldehyde. This suggests that the large reduction in number of viable cells observed when 0.5 % (w/v) sodium bisulphite was used was due to its own lethal effect on the cells rather than its inefficiency at neutralizing *ortho*-phthalaldehyde. From the neutraliser efficiency and effect tests, 0.75% azolectin in 5% Tween 80 was chosen to neutralise triclosan, cetylpyridium chloride and chlorhexidine diacetate, and 5% (w/v) glycine was selected for neutralizing *ortho*-phthalaldehyde.

3.4.3.3 Biocide killing (lethal) effect on *S. marcescens* Db11

The lethality effect of *ortho*-phthalaldehyde, triclosan, cetylpyridium chloride, and chlorhexidine diacetate on *S. marcescens* Db11, at concentrations of 100 µg/ml, 4000 µg/ml, 8 µg/ml, and 50 µg/ml respectively, was investigated over a 30 min exposure period (Figure 3.8). As both triclosan and *ortho*-phthalaldehyde were dissolved in DMSO, toxicity of 6% (v/v) DMSO, a concentration 3-fold higher than the maximum used in the investigation, was also determined in a similar manner. Results of these investigations are summarized in Table 3.9 and illustrated in Figure 3.8. It can be seen that 6% DMSO did not have a significant effect on the cells, and its reduction in number of viable cells was negligible even over long exposure time. Hence it was concluded that

DMSO at the concentrations used in this study (all of which were below 6%) did not effect the viability of *S. marcescens* Db11.

The results (Table 3.9) also showed two different patterns of killing (Figure 3.8), one for cetylpyridium chloride and chlorhexidine diacetate, and the other for *ortho*-phthalaldehyde and triclosan. The number of cells killed by chlorhexidine diacetate and cetylpyridium chloride increased as time of exposure was extended. However, triclosan and *ortho*-phthalaldehyde had maximum kill at 5 min exposure, then there was no significant change in the number of cells killed as time of exposure increased. These observations suggest that the mechanisms by which triclosan and *ortho*-phthalaldehyde enter and kill the cells may be different from those of cetylpyridium chloride and chlorhexidine diacetate. Another observation from the lethality tests results is that although on agar chlorhexidine diacetate was the most effective biocide at inhibiting the growth of *S. marcescens* Db11, it is cetylpyridium chloride that was more effective at killing the bacterium causing over 4 $\log_{10}$ reduction in the number of viable cells after 30 min of exposure at a concentration of only 8 μg/ml. Triclosan seemed to be the least effective biocide at killing *S. marcescens* Db11 as a concentration of 4000 μg/ml caused only a 3.09 $\log_{10}$ reduction in the number of viable cells after 30 min exposure time.

3.4.4 Potassium leakage from biocides-exposed *S. marcescens* Db11 cells

When the membrane of a bacterium is damaged, materials are able to leak out through the disrupted area. One of the early indicators of membrane damage is the release of potassium ions (578). This leakage usually occurs very rapidly upon exposure to a biocide active against the membrane, and is often completed within 5 min (559, 578). A positive correlation between the amount of potassium leaked and the concentration of the biocide is usually an indication that the biocide induces membrane damage. This is because low levels of the biocide would have smaller effect on the membrane, whereas higher concentrations are expected to cause greater membrane damage, allowing larger amounts of potassium to be released.

The amount of potassium leaked from a biocide-treated *S. marcescens* Db11 culture was determined at specific exposure times over a 30 min period (section 3.3.6). The organism was exposed to 8 μg/ml cetylpyridinium chloride, 20 μg/ml chlorhexidine diacetate, 4000 μg/ml triclosan, and 50 μg/ml *ortho*-phthalaldehyde. The amount of potassium present in biocide free suspensions of the test organism was also determined and taken into account during the interpretation of the results (Figure 3.9). The latter showed that there was a positive correlation between time of exposure to the biocide and the amount of potassium leaked for cetylpyridinium chloride, chlorhexidine diacetate, and to a lesser extent *ortho*-phthalaldehyde. The amount of potassium released did not increase as time of exposure to triclosan increased, and leakage reached a plateau level after 5 min exposure. These observations supports the results of the lethality tests (Table 3.9, Figure 3.8), which showed that at the tested concentrations, triclosan and *ortho*-phthalaldehyde cause the maximum amount of kill after 5 min exposure time, after which the number of cells killed did not show significant increase. The concentration of chlorhexidine diacetate used had a great lethal effect on Db11 cells and the amount of potassium released was also highest when this biocide was used. On the other hand, it is interesting that 8 μg/ml cetylpyridinium chloride, a concentration which was effective at killing the *S. marcescens* Db11 in the lethality tests, cause only small amount of potassium to be leaked from these cells.

3.4.5 Antibiotics MICs of *S. marcescens* Db11

Susceptibility of *S. marcescens* Db11 to a number of antibiotics was investigated using E-test strips (Table 3.10). Susceptibility profile of the *E. coli* NCTC 1048 reference strain was also determined, and the results are summarized in Table 3.10. Based on the British Society for Antimicrobial Chemotherapy (BSAC) MIC breakpoints for *Enterobacteriaceae* (640), the *E. coli* NCTC 1048 reference strain was sensitive to all but one of the ten antibiotics tested (tobramycin). *S. marcescens* Db11 on the other hand showed resistance to the two aminoglycosides; amikacin and tobramycin, as well as to chloramphenicol. It also showed great resistance to azithromycin with an MIC value of over 256 μg/ml. *S. marcescens* Db11 was susceptible to ceftazidime, ciprofloxacin,

imipenem, piperacillin and meropenem. When susceptibility was compared with that of the *E. coli* reference strain, *S. marcescens* Db11 was more resistant to all antibiotics tests with the exception of piperacillin where both organisms had the same MIC value. *S. marcescens* Db11 was also sensitive to 10 μg/ml gentamicin and 20 μg/ml kamamycin but was resistant to 100 units/ml polymyxin B.

Table 3.6. Biocide MIC values for S. *marcescens* Db11.

Agent	MIC µg/ml (± SD)			
	S. marcescens Db11		*E. coli* NCTC 1048	
	Agar (TSA)	**Broth (TSB)**	**Agar (TSA)**	**Broth (TSB)**
Cetylpyridinium chloride	100 (28.3)	5.5 (0.70)	12 (28.3)	2 (0.00)
Chlorhexidine diacetate	18 (2.83)	7.5 (0.70)	4 (0.00)	1 (0.00)
Triclosan	3900 (141)	Nd	0.1 (0.00)	Nd
Ortho-phthalaldehyde	3000 (283)	Nd	3000 (283)	Nd
Dimethyl sulfoxide *	Nd	10 (0.00)	Nd	Nd

SD; standard deviation, Nd; not determined.
* [Dimethyl sulfoxide] expressed in % (v/v)

Table 3.7. Neutraliser effect on cells.

Neutraliser	Number of cfu/ml *	Log_{10} reduction in cfu/ml (± SD)
None	$5.5\ 10^8$	0.00
0.5 % (w/v) sodium bisulphite	0.00	8.74 (0.00)
5% (w/v) glycine	$2.7\ 10^8$	0.30 (0.02)
0.75% azolectin in 5% Tween 80	$5.1\ 10^8$	0.03 (0.01)

* Number of cfu/ml after 5 min exposure of 100 µl of a *S. marcescens* Db11 suspension containing $5.5\ 10^8$ cfu/ml to 900 µl suspension of neutraliser. SD; standard deviation.

Table 3.8. Neutraliser efficiency tests.

Biocide	Neutraliser	Number of cfu/ml *	Log_{10} reduction in cfu/ml (± SD)
None	None	$7.5\ 10^8$	0.00
OPA	0.5 % (w/v) sodium bisulphite	0.00	8.87 (0.00)
OPA	5% (w/v) glycine	$3.1\ 10^8$	0.38 (0.03)
OPA	0.75% azolectin in 5% Tween 80	$3.3\ 10^7$	1.35 (0.07)
TRI	0.75% azolectin in 5% Tween 80	$6.8\ 10^8$	0.04 (0.20)
CPC	0.75% azolectin in 5% Tween 80	$5.5\ 10^8$	0.13 (0.18)
CHX	0.75% azolectin in 5% Tween 80	$5.5\ 10^8$	0.13 (0.21)

* Number of cfu/ml after addition of 100 µl of a *S. marcescens* Db11 suspension containing $7.5\ 10^8$ cfu/ml to a 900 µl mixture of biocide and neutraliser (1:8 v/v), and left for 5 min.
TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate, SD; standard deviation.

Table 3.9. Lethal effect of biocides on *S. marcescens* Db11.

Exposure time (min)	Log_{10} reduction in cfu/ml (± SD)				
	CPC 8 µg/ml	**CHX 50 µg/ml**	**OPA 100 µg/ml**	**TRI 4000 µg/ml**	**DMSO 6% (v/v)**
0	0.00	0.00	0.00	0.00	0.00
5	2.37 (0.19)	2.19 (0.28)	3.12 (0.33)	3.04 (0.34)	0.03 (0.03)
10	3.28 (0.20)	2.87 (0.10)	3.22 (0.09)	3.07 (0.06)	0.06 (0.04)
20	3.58 (0.39)	4.50 (0.28)	3.30 (0.08)	3.09 (0.37)	0.04 (0.23)
30	4.42 (0.22)	5.30 (0.34)	3.32 (0.20)	3.09 (0.58)	0.04 (0.04)

TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate, DMSO; dimethyl sulfoxide, SD; standard deviation

Table 3.10. Antibiotic MIC values for *S. marcescens* Db11.

Antibiotic	BSAC MIC breakpoint (µg/ml)		*E. coli* NCTC 1048		*S. marcescens* Db11	
	Susceptible ≤	Resistant ≥	MIC (µg/ml) ± SD	Interpretation*	MIC (µg/ml) ± SD	Interpretation*
Amikacin	4	8	2 ± 0.00	S	8 ± 0.00	R
Azithromycin	-	-	4.6 ± 0.00	-	>256 ± 0.00	-
Ceftazidime	2	4	0.5 ± 0.00	S	0.71 ± 0.31	S
Chloramphenicol	8	16	4 ± 0.00	S	24 ± 8.00	R
Ciprofloxacin	1	2	0.016 ± 0.00	S	0.17 ± 0.04	S
Imipenem	4	8	0.25 ± 0.00	S	0.59 ± 0.09	S
Piperacillin	16	32	2 ± 0.00	S	2 ± 0.00	S
Tobramycin	1	2	2 ± 0.00	R	25.1 ± 8.55	R
Trimethoprim/ sulfamethoxazole**	-	-	0.094 ± 0.00	-	0.65 ± 0.13	-
Meropenem	4	8	0.05 ± 0.01	S	0.12 ± 0.05	S

* Susceptibility based on BSAC MIC breakpoints for *Enterobacteriaceae* (640).
** Trimethoprim/sulfamethoxazole (1/19).
SD; standard deviation. Susceptible phenotype is highlighted in red, and resistance in blue.

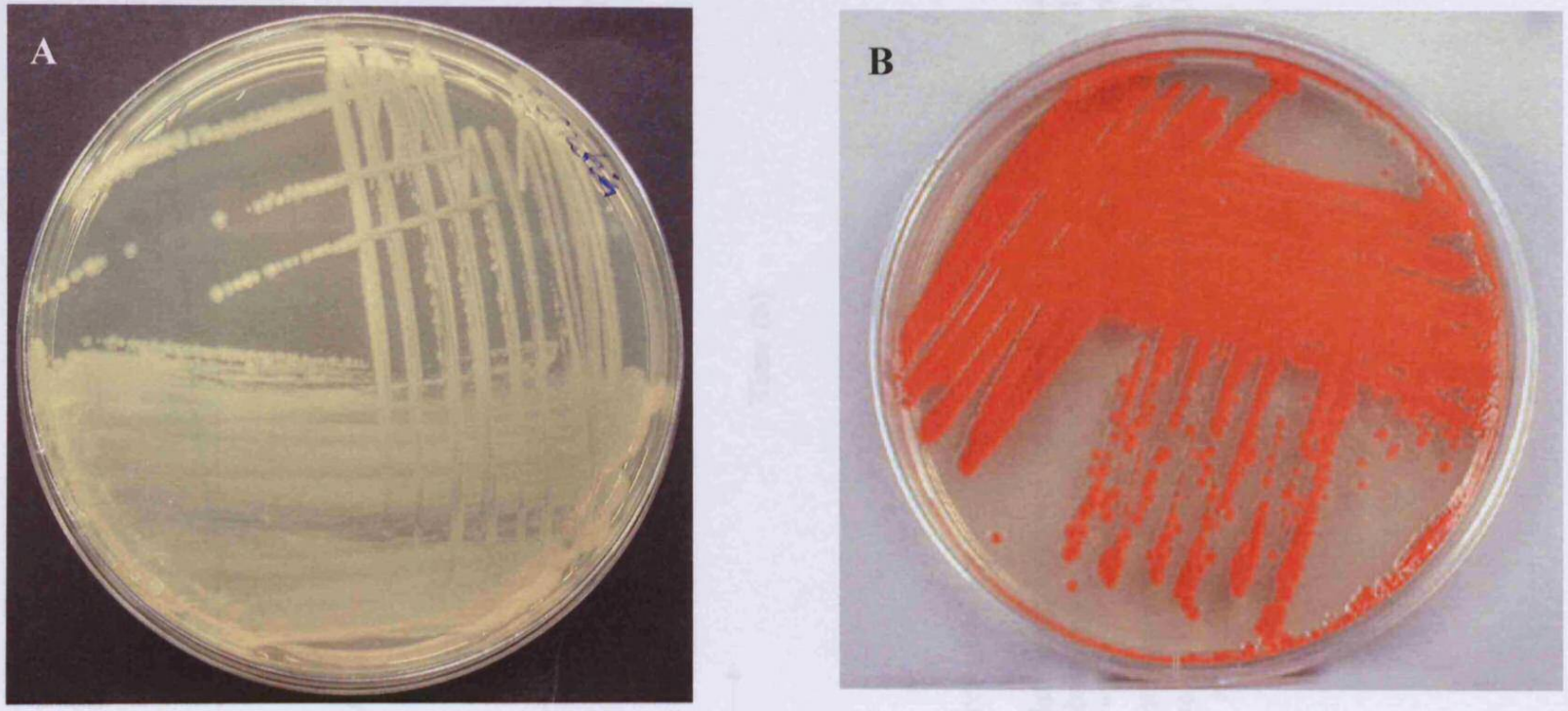


Figure 3.4. Colony morphology of chromogenic *S. marcescens* and non-pigmented *S. marcescens* Db11.

S. marcescens forms smooth mucoid colonies which have entire margins and umbonate elevation when grown on TSA. Some strains of *S. marcescens* (B) (511) are chromogenic and produce the red pigment prodigiosin, whereas others such as strain Db11 (A) are non-pigmented.

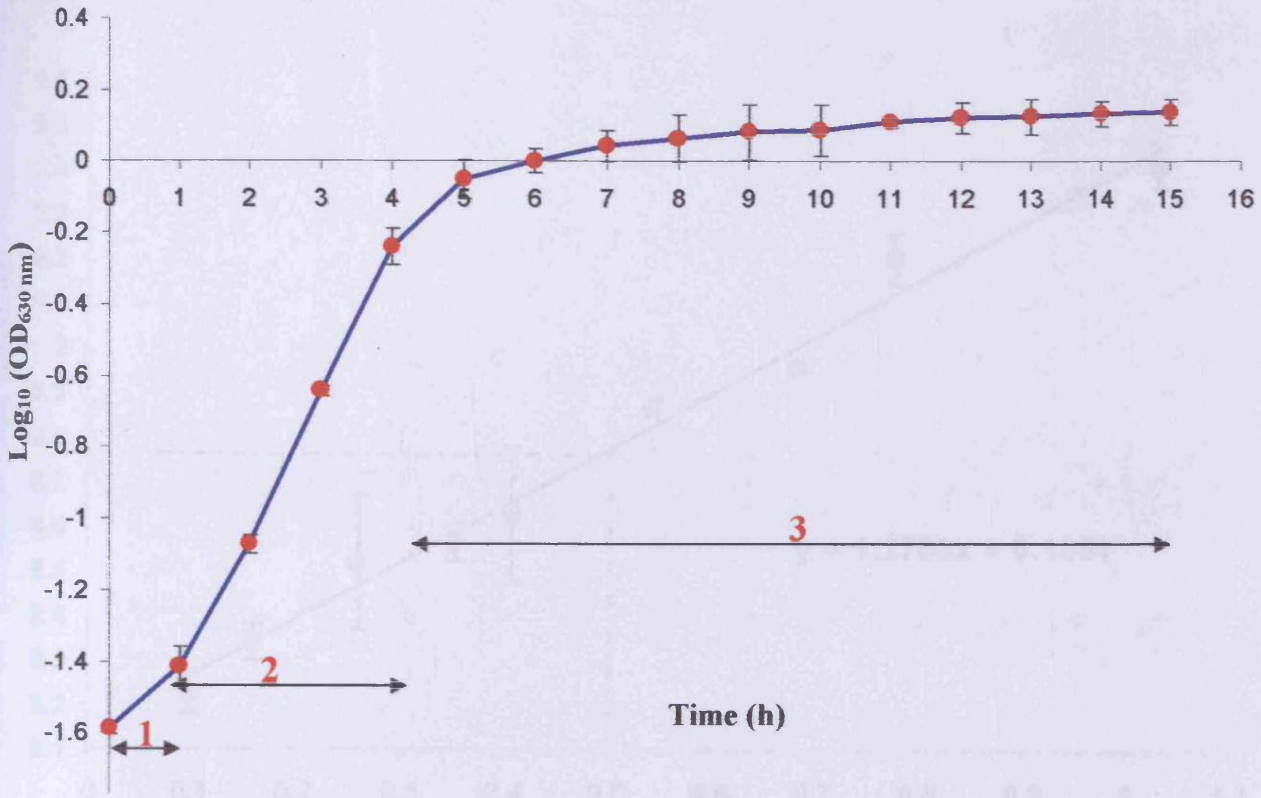


Figure 3.5. *S. marcescens* Db11 growth curve

Graph showing the relationship between $\log_{10}$ of the OD_{630nm} of a *S. marcescens* Db11 culture and time. The bacterium was grown in TSB at 37^0C over a period of 15 h. 1, 2, and 3 represent the "lag", "exponential (log)" and "stationary" phases of growth respectively. The points represent mean values and the error bars represent standard error of the means. The culture had a "log" phase that extended over 4 h with a specific growth rate $\mu = 0.9\ h^{-1}$ and a culture doubling time g = 0.77 h.

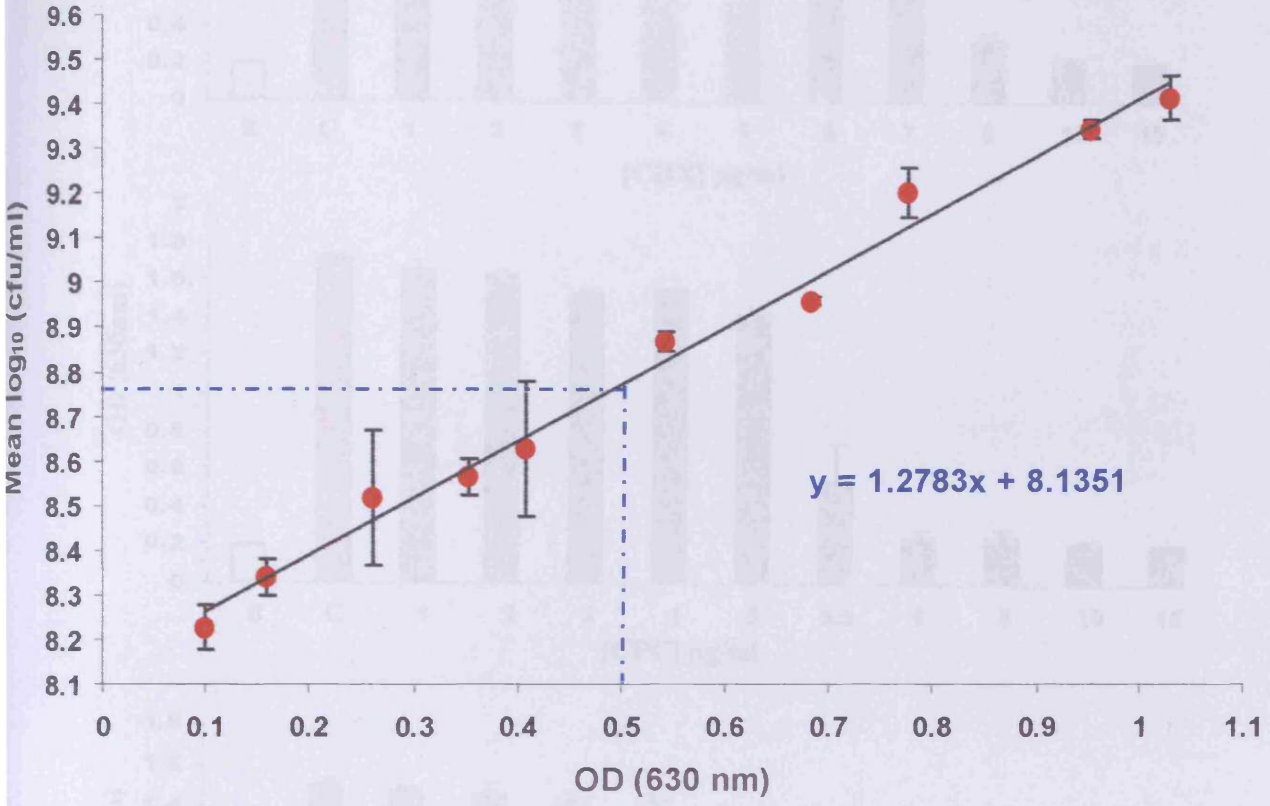


Figure 3.6. Relationship between OD_{630nm} and viable count for *S. marcescens* Db11 culture

Line of best fit showing the relationship between $\log_{10}$ (cfu/ml) of a *S. marcescens* Db11 culture and its OD_{630nm} values. The points represent mean values and the error bars represent standard error of the means. The equation for the line of best fit is shown on the graph. It can be seen that a *S. marcescens* Db11 culture of an OD_{630nm} value of 0.5 has approximately 6.10^8 cfu/ml of bacteria.

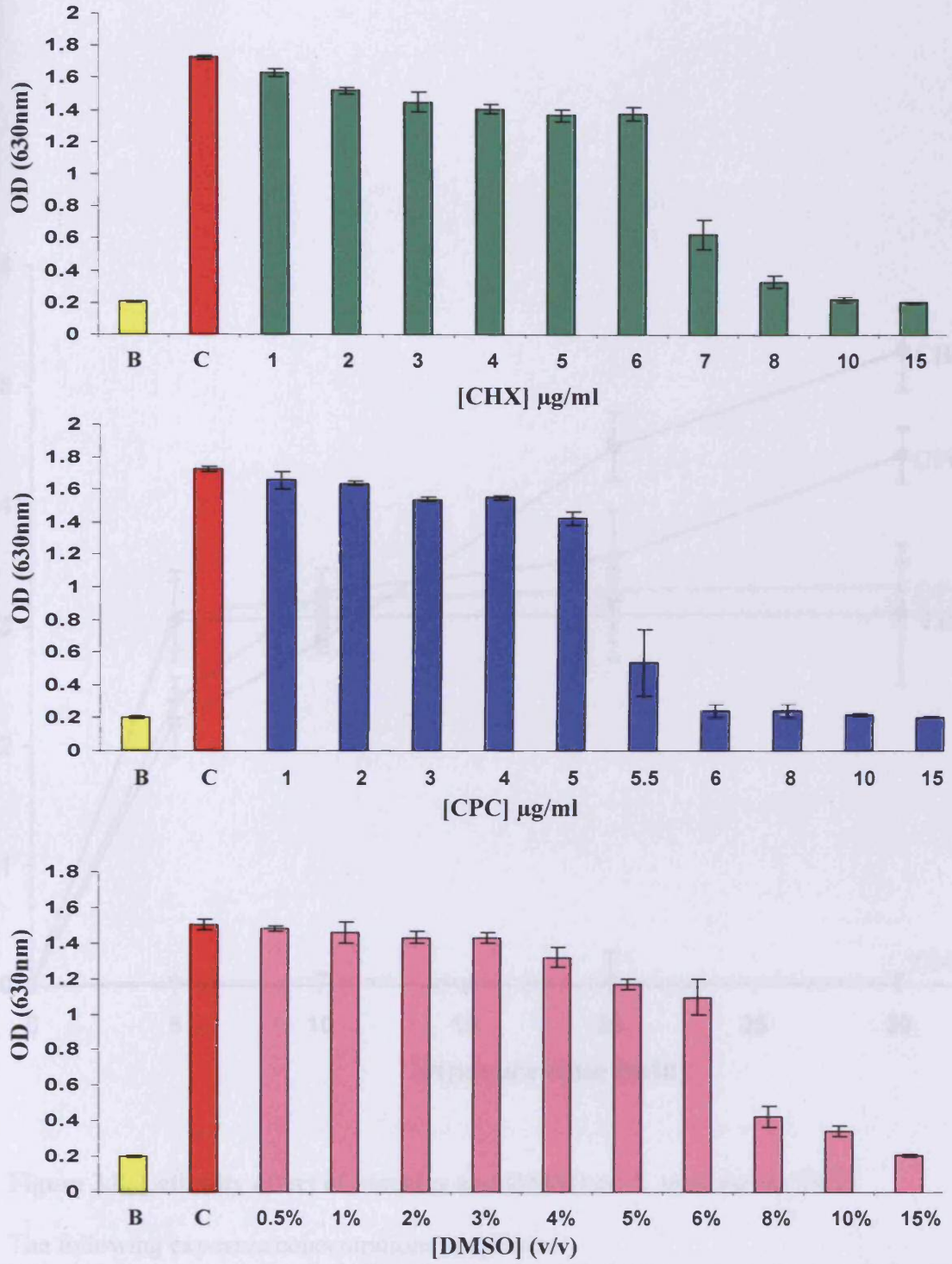


Figure 3.7. Broth MICs of cetylpyridinium chloride, chlorhexidine diacetate, and DMSO for *S. marcescens* Db11.

MICs were determined in TSB broth supplemented with the appropriate concentration of agents. OD_{630nm} values were generated after 1 day incubation at 37^0C. The bars represent mean values and the error bars represent standard error of the means. CHX; chlorhexidine diacetate, CPC; cetylpyridinium chloride, DMSO; dimethyl sulfoxide, B; blank, C; control.

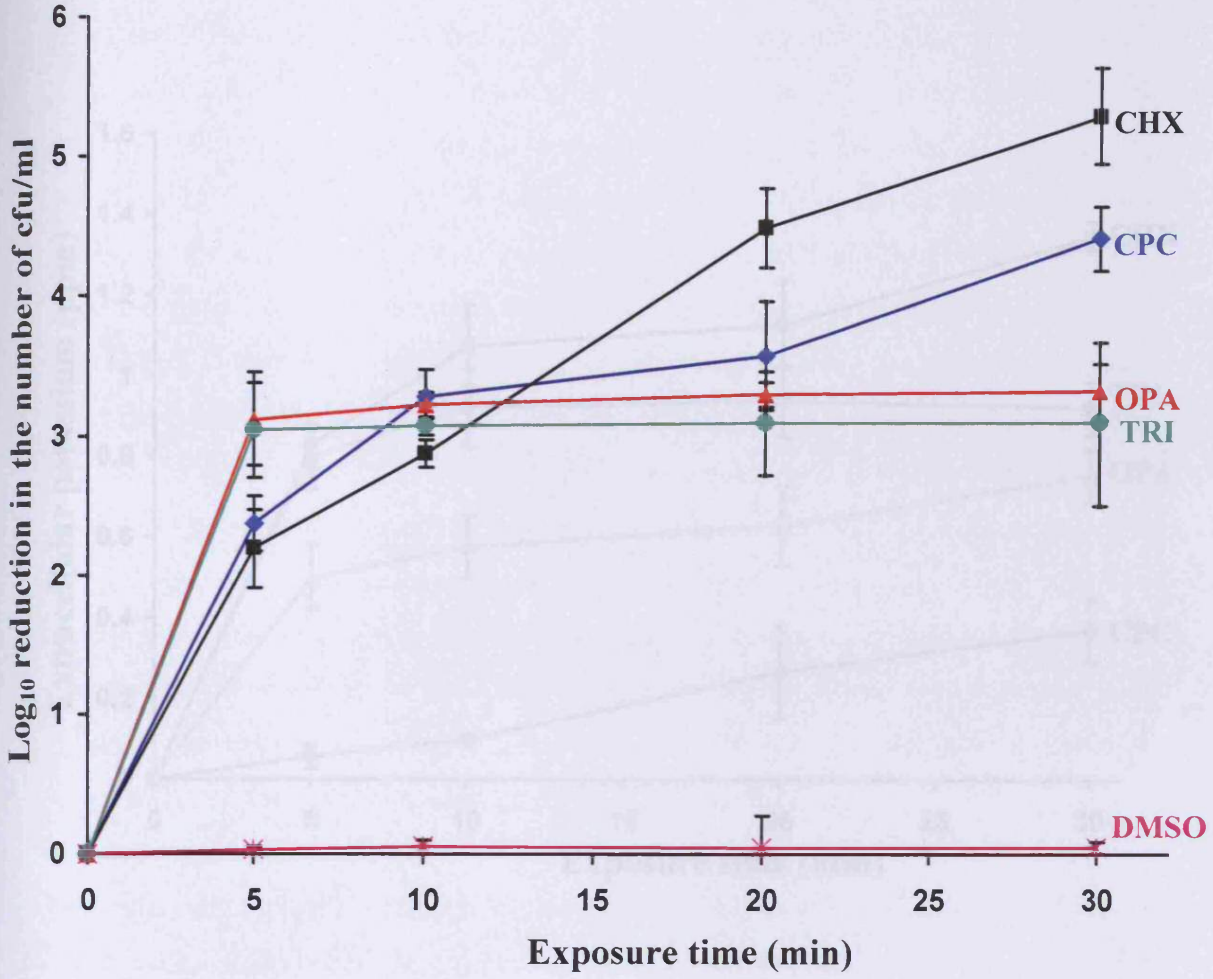


Figure 3.8. Lethality effect of biocides and DMSO on *S. marcescens* Db11

The following exposure concentrations are plotted:
TRI; triclosan at 4000 µg/ml, OPA; *ortho*-phthalaldehyde at 100 µg/ml, CPC; cetylpyridinium chloride at 8 µg/ml, CHX; chlorhexidine diacetate at 50 µg/ml. DMSO; dimethyl sulfoxide at 6% (v/v). The points represent mean values and the error bars represent standard error of the means.

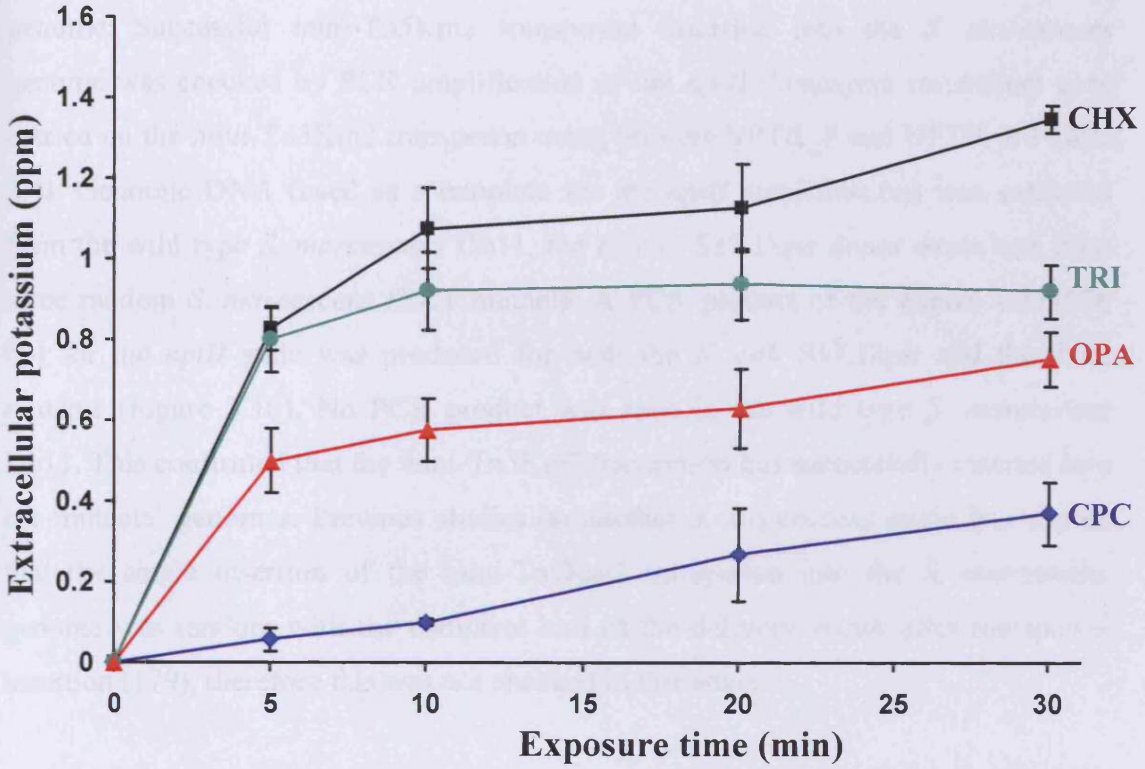


Figure 3.9. Potassium leakage from biocide-treated *S. marcescens* Db11 cultures

Leakage after exposure to he following is shown:
TRI; triclosan at 4000 µg/ml, OPA; *ortho*-phthalaldehyde at 50 µg/ml, CPC; cetylpyridinium chloride at 8 µg/ml, CHX; chlorhexidine diacetate at 20 µg/ml. The points represent mean values and the error bars represent standard error of the means.

3.4.6 Generation of *S. marcescens* Db11 mutants and their screening for biocide susceptibility

Random transposon mutagenesis using the mini-Tn*5*Km2 transposon was used to generate a *S. marcescens* Db11 mutant library (section 3.3.11). Mating between the wild type *S. marcescens* Db11 and the *E. coli* S17.1λpir containing the delivery vector pUTmini-Tn*5*Km2 was used to deliver the transposon into the *S. marcescens* Db11 genome. Successful mini-Tn*5*Km2 transposon insertion into the *S. marcescens* genome was checked by PCR amplification of the *nptII* (knamycin resistance) gene carried on the mini-Tn*5*Km2 transposon using primers NPTII_F and NPTII_R (Table 3.4). Genomic DNA (used as a template for the *nptII* amplification) was extracted from the wild type *S. marcescens* Db11, the *E. coli* S17.1λpir donor strain and from three random *S. marcescens* Db11 mutants. A PCR product of the expect size (676 bp) for the *nptII* gene was produced for both the *E. coli* S17.1λpir and the three mutants (Figure 3.10). No PCR product was seen in the wild type *S. marcescens* Db11. This confirmed that the mini-Tn*5*Km2 transposon has successfully inserted into the mutants' genomes. Previous studies on another *S. marcescens* strain had shown that the single insertion of the mini-Tn*5*Km2 transposon into the *S. marcescens* genome was random with the complete loss of the delivery vector after transposon insertion (179), therefore this was not checked in this study.

The conjugative transfer of the pUTmini-Tn*5*Km2 delivery vector into *S. marcescens* Db11 and subsequent transposon insertion into its genome was performed so that only *S. marcescens* Db11 mutants containing the mini-Tn*5*Km2 were isolated onto the selective TSA plates containing 100 µg/ml KAN and 100 units/ml PXB. The *S. marcescens* Db11 recipient strain was not able to grow on TSA containing 100 µg/ml KAN, but was resistant to 100 units/ml PXB. Similarly, the *E. coli* S17.1λpir donor strain was resistant to 100 µg/ml KAN, due to the presence of the pUTmini-Tn*5*Km2 plasmid, but was sensitive to 100 units/ml PXB. Both donor and recipient strains did not grow onto the selective TSA plates when plated individually as controls. The *S. marcescens* Db11 transposon mutants on the other hand, were able to grow onto the selective plates because of the presence of the mini-Tn*5*Km2 (enabling them to grow on agar containing 100 µg/ml KAN), and their natural resistance to PXB (enabling them to grow on agar containing 100 units/ml PXB). These controls did not eliminate

the possibility of contamination of the selected mutants either by a mutated donor *E. coli* S17.1λpir or another naturally resistant organism. RAPD analysis (below) was used to eliminate this possibility.

A *S. marcescens* Db11 mutant bank containing 6000 mutants was screened on agar at the following biocide concentrations to isolate biocide sensitive or resistant mutants. Triclosan at 2500, 3000, 3500, and 4000 μg/ml, *ortho*-phthalaldehyde at 2400, 2800, 3000 and 3200 μg/ml, cetylpyridium chloride at 50, 70, 90, and 110 μg/ml, and chlorhexidine diacetate at 10, 14, 18, and 26 μg/ml. Eighty mutants showed varying degrees of sensitivity to at least one of the four biocides; of these, 26 mutants that showed the clearest phenotype were isolated and used for further analysis. Two randomly selected mutants which showed no change in their biocide susceptibility compared to the wild type were also selected and used as controls for further investigations. To make sure that the 26 biocide mutants and the two controls chosen for further investigation were actually *S. marcescens* Db11, RAPD analysis was performed on the *S. marcescens* Db11 transposon mutants.

The PCR fingerprinting analysis used primer 270 and 272 (section 3.3.10, Table 3.4) both of which were already used to produce discriminatory polymorphisms from CF isolates of *P. aeruginosa* and *B. cepacia* (644, 645). Initially the analysis used primer 270 and the results were later confirmed using primer 272. DNA from all the 26 *S. marcescens* Db11 biocide mutants and the two controls was extracted using chelex method (section 2.3.11.1.1), and used as template for the RAPD PCR reactions. DNA from parent *S. marcescens* Db11 was used as a positive control. As one of the main possible sources of contamination of the mutants was the donor *E. coli* S17.1λpir, DNA from the latter was used as a negative control. The RAPD results obtained with primer 270 (Figure 3.11), showed that all the transposon mutants had the same RAPD fingerprint profiles as the parent *S. marcescens* Db11. RAPD profiles obtained with DNA from *E. coli* S17.1λpir were clearly different. These results were confirmed by the RAPD fingerprints obtained using primer 272, concluding that all the transposon mutants investigated in this study were derived only from *S. marcescens* strain Db11.

3.4.7 Mutants growth rates

All the isolated *S. marcescens* Db11 mutants grew well on agar or in broth media with or without kanamycin. However, for a minority of mutants, visually there was noticeable difference in their growth when compared to the wild type. To determine whether the mutants were as growth fit as the wild type, the mean growth rates of the 26 biocide mutants and the two controls were compared to that of the wild type *S. marcescens* Db11 by t-test at the 95% confidence level. The growth rates were calculated from growth curves generated using an automated plate reader. The mean growth rate of the reference *E. coli* NCTC 1048 strain was also calculated and included in the comparison. The results (Table 3.11) showed that except for 6 mutants, the mean growth rate of the mutants was not significantly different from that of the wild type *S. marcescens* Db11. The mean growth rates for mutants N2-F3, 22-D5, N6-B2, N5-G1, N5-B5 and N5-B6, were significantly different from the wild type, and so was that of the reference strain *E. coli* NCTC 1048.

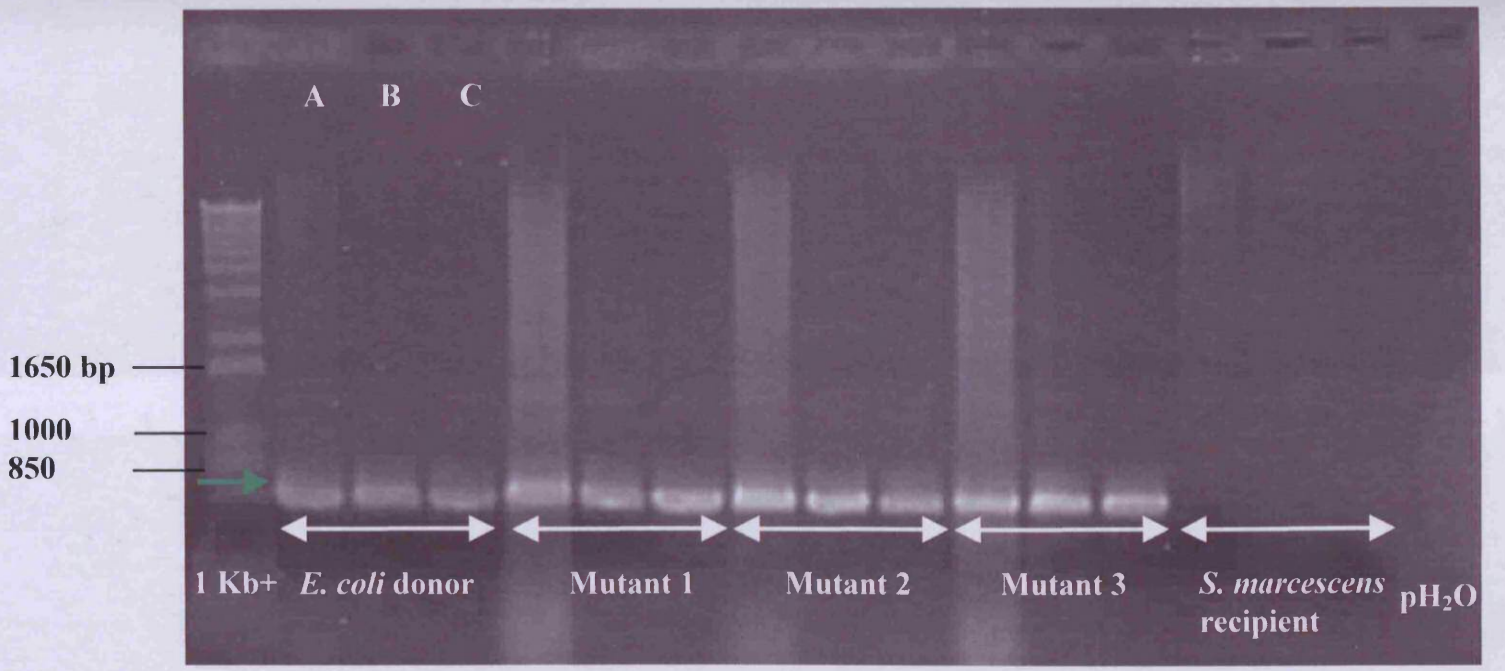


Figure 3.10. Confirmation of mini-Tn*5*Km2 insertion into *S. marcescens* Db11 genome using PCR

A 1.5% agarose gel showing the *nptII* gene PCR products obtained with both the *E. coli* S17.1λpir donor and the *S. marcescens* Db11 recipient strains, and three random *S. marcescens* Db11 mutants: 1, 2, and 3 resulting from the mating between donor and recipient. *S. marcescens* Db11 and sterile polished water (pH_2O) were used as negative controls, while the *E. coli* S17.1λpir donor strain was used as a positive control. The 1-Kb+ DNA ladder was the size marker. A, B, and C, respectively represent three different DNA concentrations of the strains used (≈50, ≈5, and ≈0.5 ng/µl). Strong PCR products of the expected size (676 bp) were obtained for both the positive control and all the *S. marcescens* Db11 mutants. No PCR product was observed in the recipient *S. marcescens* Db11 strain.

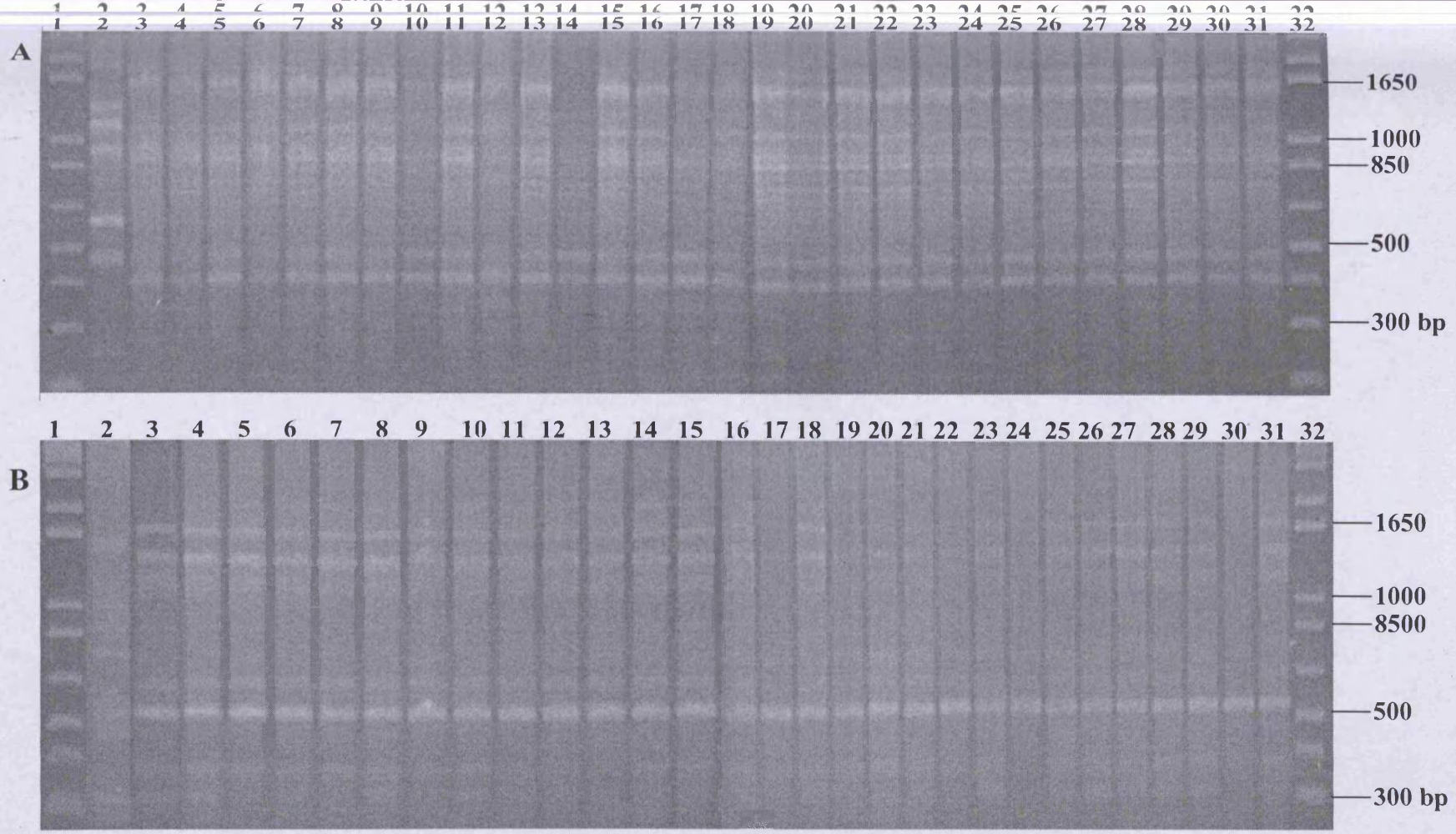


Figure 3.11. Randomly amplified polymorphic DNA (RAPD) from *S. marcescens* Db11 mutants

DNA from wild type *S. marcescens* Db11 (lane 3) and *S. marcescens* Db11 mutants (lanes 4-31), along with DNA from *E. coli* S17.1λpir (lane 2) were used as template for amplification with primers 270 (A) and 272 (B). Lanes 1 and 32 represent 1-Kb+ DNA ladder, lanes 4-31 represent respectively the following mutants: 10-B6, 10-E7, 9-D5, 3-A4, 19-D3, 3-F2, 9-D10, 11-B8, 12-F6, 8-C7, 7-C10, 18-A3, 22-D5, 27-B8, N6-B2, N5-B5, N5-G6, N5-B6, N2-F3, N3-B8, N5-D9, N2-F1, N4-F6, N2-B3, N2-A8, N1-C5, N3-C10 and N5-G1. *E. coli* S17.1λpir had different amplification patterns from those of the wild type *S. marcescens* Db11. All mutants had the same patterns as the wild type with both primers.

Table 3.11. Growth rates of *S. marcescens* Db11 and its biocide mutants.

Organism	Mean μ (h^{-1})	SE mean	P-value
S. marcescens Db11	0.9095	0.0125	-
E. coli NCTC 1048*	1.014	0.0021	0.036
S. marcescens mutants			
Mutants un-altered in growth rate			
3-F2	0.9095	0.0125	1.000
7-C10	0.8150	0.0050	0.090
12-F6	0.8710	0.0260	0.409
27-B8	0.8835	0.0385	0.637
8-C7	0.8835	0.0385	0.637
19-D3	0.9735	0.0005	0.123
N1-C5	0.8150	0.0050	0.090
N2-F1	0.8450	0.0000	0.123
N2-B3	0.7300	0.0130	0.064
3-A4	0.8715	0.0005	0.202
10-B6	0.8585	0.0385	0.427
N2-A8	0.7940	0.0260	0.156
9-D10	0.8715	0.0005	0.202
N3-B8	0.8525	0.0075	0.159
10-E7	0.8065	0.0385	0.238
N5-G6	0.8250	0.0050	0.101
N4-F6	0.7940	0.0260	0.516
18-A3	0.7945	0.0005	0.069
N5-D9	0.8070	0.0130	0.111
9-D5	0.9095	0.0385	1.000
11-B8	0.8580	0.0130	0.214
N3-C1	0.9309	0.0330	0.349
Mutants altered in growth rate*			
N2-F3	0.5760	0.0130	0.034
N6-B2	0.5425	0.0255	0.031
N5-G1	0.8325	0.0125	0.049
22-D5	0.5250	0.0130	0.030
N5-B5	0.7435	0.0005	0.048
N5-B6	0.6020	0.0130	0.037

μ; exponential growth rate, SE; standard error, P-value; P-value for the two-sample t-test. *; growth rates which were statistically significantly different from that of the wild type *S. marcescens* Db11 are highlighted (red).

3.4.8 Determination of biocide agar MICs for the *S. marcescens* Db11 mutants

Biocides agar MICs were determined using TSA plates containing the appropriate concentrations of biocides, inoculated with bacterial cultures ($\approx 10^8$ cfu/ml) using a multi-inoculator, and checking for inhibition of growth after overnight incubation at 37^0C. Agar MICs of the 26 isolated biocide mutants were determined for the four biocides, the results are summarized in Table 3.12. MICs for the *E. coli* NCTC 1048 reference strain and two *S. marcescens* mutant controls were also determined. Out of the 26 biocide mutants investigated 30% were chlorhexidine diacetate sensitive, 53% were cetylpyridium chloride sensitive, 42% were triclosan sensitive and 42% were *ortho*-phthalaldehyde sensitive. The proportion of resistant mutants was 23% for chlorhexidine diacetate, 11% for cetylpyridium chloride and *ortho*-phthalaldehyde, and 7% for triclosan. It was noticeable that the most dominant sensitivity was to cetylpyridium chloride whereas resistance to chlorhexidine diacetate was the most common resistance phenotype. Furthermore, 84% of the mutants showed some sensitivity or resistance to more than one biocide.

The other general observation from the data was that the relative increase in sensitivity or resistance to biocides in the mutants was low in most mutants. Mutants 9-D5 and 3-A4 (Table 3.12) showed the highest increase in sensitivity of 40% to cetylpyridium chloride, whereas the highest increase in resistance was seen in mutant N3-B8 (Table 3.12) which showed a 100% increase in resistance to the above biocide compared to the wild type. The biocide MICs for the two control mutants were similar to those of the wild type. Only changes in triclosan MICs for the *E. coli* NCTC 1048 reference strain, mutant N5-B6, and N2-F3 were statistically significant at 95% confidence level. However, it was noted that biocide MICs changes in the mutants were reproducible and constant in all our replica experiments. To investigate this further, the ratios between the biocide MIC values of the mutants and the wild type were determined. Ratios were calculated for both the maximum and the minimum MIC values determined for each biocide from our replica experiments (Table 3.13).

A number of observations could be made regarding the results:

1) The ratios, as expected, revealed that the largest difference in MIC values between the mutants and the wild type was in mutants 9-D5 and 3-A4 for the sensitive phenotype and N3-B8 and 19-D3 for resistance phenotype.
2) The ratio values for many mutants were close to 1, the largest deviation from the latter number did not exceed 0.55 for susceptible phenotypes and 1.83 for resistance.
3) The ratio values were reproducible and constant whether it was the maximum or the minimum biocide MIC values used to generate them.

These observations, suggested that although the changes in biocide resistance or susceptibility in the mutants were small, these changes were genuine, and reproducible. Hence the mutants phenotypes determined from the agar biocide MICs were taken as the basis for further investigations and the results from further tests, including biocide broth MICs (Table 3.14 and 3.15), suspension tests (Table 3.16) and potassium leakage experiments (Table 3.17), were used to confirm these phenotypes using different assays.

3.4.9 Biocide growth inhibition of *S. marcescens* Db11 mutants in broth

Inhibition of maximal growth in broth for both chlorhexidine diacetate and cetylpyridium chloride was determined by inoculating 96-well plates filled with TSB containing the appropriate concentration of biocide tested, with test cultures ($\approx 10^8$ cfu/ml) using a multi-inoculator. The plates were then incubated at 37^0C overnight, and inhibition of growth was determined by reading the OD_{630nm} of cultures using an automated plate reader and comparing the results with controls grown in the absence of the biocide. The mean OD_{630nm} values were calculated from replica for each tested mutant and were compared to that of the wild type using t-test. The biocide concentrations tested were 5 and 6 μg/ml for cetylpyridium chloride and chlorhexidine diacetate respectively.

In the control plates, which did not contain biocides, all mutants grew to OD_{630nm} values comparable to that of the wild type with no statistically significant differences noted. The wild type *S. marcescens* Db11 grew to an OD_{630nm} value of 1.42 in TSB containing 5 μg/ml cetylpyridium chloride, while the control grew to an OD_{630nm} value of 1.7. The same biocide concentration had varying effect on the *S. marcescens* Db11 mutants (Table 3.14). Statistical analysis showed that after overnight growth at 37^0C, cultures of mutants 3-A4, 10-B6, 22-D5, 8-C7, 10-E7, 9-D5, 18-A3, N6-B2, N4-F6, N2-A8, 9-D10, and N5-G1, all grew to OD_{630nm} values that were significantly lower than that of the wild type culture grown in same cetylpyridium chloride concentration. Similarly the analysis showed that cultures of mutants 19-D3, N3-B8, and N5-B5 grown at same biocide concentration as the wild type, grew to OD_{630nm} values that were significantly higher than that of the wild type. The OD_{630nm} values of the cultures of the remaining mutants were not different from that of the wild type.

Results of the chlorhexidine diacetate broth growth inhibition (Table 3.15) showed that *S. marcescens* Db11 grew to an OD_{630nm} value of 1.38 in TSB containing 6 μg/ml chlorhexidine diacetate, while the control (not exposed to the biocide) grew to an OD_{630nm} value of 1.7. The same biocide concentration had varying effect on the *S. marcescens* Db11 mutants (Table 3.15). Statistical analysis showed that after overnight growth at 37^0C, cultures of mutants 12-F6, 18-A3, 9-D5, N4-F6, N2-A8, N5-G1, N6-B2, and N2-F1, all grew to OD_{630nm} values that were statistically significantly lower than that of the wild type culture grown in same chlorhexidine diacetate concentration. Similarly statistical analysis showed that cultures of mutants 19-D3, N3-B8, and N5-G6 grown at same biocide concentration as the wild type, grew to OD_{630nm} values that were statistically significantly higher than that of the wild type. The OD_{630nm} values of the cultures of the remaining mutants were not different from that of the wild type.

3.4.10 Suspension (killing) tests on *S. marcescens* Db11 mutants

The lethal effects of triclosan, *ortho*-phthalaldehyde, cetylpyridium chloride, and chlorhexidine diacetate on *S. marcescens* Db11 and its mutants were determined using suspension tests (section 3.3.5.3). The viable counts of cultures subjected to a specific concentration of a biocide were determined at different times of exposure. These were 10 min for cultures exposed to 8 μg/ml cetylpyridinium chloride or 20 μg/ml chlorhexidine, and 5 min for cultures exposed to 4000 μg/ml triclosan or 50 μg/ml *ortho*-phthalaldehyde. The viable counts results were used to calculate the $\log_{10}$ reduction in number of cells caused by the biocide at the corresponding exposure times, hence determining the biocide's lethal effect. The biocides' lethal effects on the mutants were statistically compared to those on the wild type *S. marcescens* Db11. The killing effect of the biocides was also determined for the *E. coli* NCTC 1048 reference strain.

The results (Table 3.16) showed that all four biocides were effective at killing the *E. coli* NCTC 1048 reference strain, and with the exception of *ortho*-phthalaldehyde, they all lead to the complete killing of the *E. coli* NCTC 1048 cells at the end of the exposure time. All of biocides had a greater killing effect on the reference *E. coli* NCTC 1048 compared to the wild type. When subjected to 8 μg/ml cetylpyridinium chloride for 10 min, there was a 3.13 $\log_{10}$ reduction in number of cells in the wild type *S. marcescens* Db11 culture. The same concentration of the above biocide for 10 min, caused a greater $\log_{10}$ reduction in number of cells in cultures of mutants 10-B6, 10-E7, 9-D5, 3-A4, 9-D10, 22-D5, N4-F6, N5-G1 and 18-A3 (Table 3.16). This suggests that the biocide had a greater lethal effect on these mutants than the wild type. Cetylpyridinium chloride had a less significant killing effect on mutants 19-D3, N5-B5, and N3-B8 compared to the wild type (Table 3.16).

Similarly, 10 min exposure to 20 μg/ml chlorhexidine diacetate had a greater lethal effect on mutants 9-D5, 12-F6, 7-C10, 18-A3, 22-D5, 27-B8, N4-F6, N2-B3, N2-A8, N5-G1, and N2-F1 and achieved lower killing in mutants 3-A4, 19-D3, N5-G6, and N3-B8 compared to the wild type (Table 3.16). The lethal effect of exposure to 4000 μg/ml

triclosan for 5 min was higher in mutants 3-A4, 9-D10, 12-F6, 7-C10, 22-D5, N6-B2, N5-B5, N5-G6, N3-B8, N5-D9, and N2-B3, and lower in mutants 10-B6, 8-C7, 18-A3, 27-B8, and N4-F6, compared to the wild type (Table 3.16). The lethal effect of exposure to 50 μg/ml *ortho*-phthalaldehyde for 5 min was higher in mutants 10-E7, 3-F2, 11-B8, 12-F6, N6-B2, N5-B5, N5-G6, N2-F3 and N2-B3, and lower in mutants 10-B6, 19-D3, 8-C7, 18-A3, and N2-A8, compared to the wild type (Table 3.16). All biocides had a greater killing effect on the reference *E. coli* NCTC 1048 compared to the wild type.

3.4.11 Potassium leakage experiment on *S. marcescens* Db11 mutants

To determine differences in biocide-induced membrane damage on *S. marcescens* Db11 mutants, the amount of potassium leaked from these mutants when subjected to a particular biocide was measured (section 3.3.6). The results (Table 3.17) were statistically compared with those generated using the wild type *S. marcescens* Db11. Potassium leakage from the *E. coli* NCTC 1048 reference strain was also determined and used for comparative purposes. Exposure to 8 μg/ml cetylpyridinium chloride for 10 min caused the highest proportion of mutant (64%) to leak larger amounts of potassium than the wild type. This proportion was 42% for mutants exposed to 4000 μg/ml triclosan, 32% for mutants exposed to 50 μg/ml *ortho*-phthalaldehyde for 5 min, and 28% after exposure to 20 μg/ml chlorhexidine diacetate for 10 min. The results were reversed for the percentage of mutants leaking smaller amounts of potassium than the wild type when exposed to the biocides. Exposure to 20 μg/ml chlorhexidine diacetate for 10 min had the highest percentage of such mutants (21%), followed by 8 μg/ml cetylpyridinium chloride (14%), 50 μg/ml *ortho*-phthalaldehyde (7%) and finally 4000 μg/ml triclosan (3%). As potassium leakage is a measure of biocide-induced membrane damage, it was expected that the results of this experiment will mirror those obtained from the suspension tests (Table 3.16). Indeed for most mutants the results of potassium leakage experiments were a mirror to those of the suspension tests and in many cases to those generated by agar and broth MICs. Moreover, in general, mutants which were resistant to a particular biocide leaked less potassium than the wild type when it was exposed to the same biocide. Sensitive mutants leaked more potassium than the wild type when exposed to the

corresponding biocide. Such correlation between the level of resistance to a particular biocide and potassium leakage from cells exposed to this biocide has been reported in *S. marcescens* (582)

However, for some mutants such as mutant 8-C7 this was not the case. In the potassium leakage experiments, the latter mutant was shown to leak larger amounts of potassium compared to the wild type when individually exposed to all four biocides. This was not expected as the same mutant showed increased resistance to both chlorhexidine diacetate and triclosan on agar (Table 3.12) and was more resistant than the wild type to the killing effect of the above two biocides as well as *ortho*-phthalaldehyde (Table 3.16). The fact that the negative control suspension of mutant 8-C7 (mutant incubated in biocide-free saline solution) had an unusually higher potassium concentration, may explain the above result.

3.4.12 Antibiotic susceptibility profiles of the *S. marcescens* mutants

Antibiotic MICs for *S. marcescens* Db11 transposon mutants were determined using E-test on TSA (section 3.3.7), and the results were statistically compared to those of the wild type (Table 3.18). All mutants showed changes in their antibiotic phenotype compared with the wild type, and with the exception of the control mutant N1-C5, all remaining mutants had at least two or more changes in their antibiotic susceptibility profiles compared with the wild type. Moreover, 90% of the mutants showed increased susceptibility to at least one antibiotic. In addition, a high number of mutants (70%) also showed increased resistance to at least one antibiotic as well. Mutants 27-B8 and 18-A3 in particular, respectively demonstrated increased resistance to 8 and 7 out of 10 antibiotics tested. The most common antibiotic to have decreased MICs was meropenem with 65% of the susceptible mutants possessing increased sensitivity to this antibiotic. On the other hand, amikacin was the most common antibiotic to have increased MICs; 52% of all resistant mutants possessed increased tolerance to this antibiotic. No mutant showed increased susceptibility to ceftazidime and only 3% of all mutants showed decreased MICs to chloramphenicol or trimethoprim/sulfamethoxazole. Increased resistance to

azithromycin was not seen as the wild type was already resistant to the maximum amount of this antibiotic assayable by E-test (MIC >256 (μg/ml).

There was a noticeable correlation between the mutants' sensitivities to the two aminoglycosides, amikacin and tobramycin, as all the mutants which showed increased resistance to tobramycin also showed increased resistance to amikacin. Moreover, nearly 80% of the mutants with increased susceptibility to tobramycin, were also sensitive to amikacin. The correlation was not as clear cut with the two carbapenems, imipenem and meropenem. Increased sensitivity to meropenem correlated with increased sensitivity to imipenem as all imipenem-susceptible mutants were also susceptible to meropenem. However, this correlation was not apparent with the resistant phenotype, and only 33% of imipenem-resistant mutants were also resistant to meropenem

In order to determine if there was a correlation between biocide sensitivity and antibiotic susceptibility changes, the percentages of mutants with a particular biocide susceptibility phenotype showing increased or decreased susceptibility to particular antibiotics were determined. Increased susceptibility to amikacin and tobramycin were the most common phenotypes amongst the chlorhexidine diacetate-sensitive mutants. Of these mutants, 75% showed decreased MICs to amikacin and nearly 63% were sensitive to tobramycin. Meropenem sensitivity was the most common phenotype amongst chlorhexidine diacetate-resistant mutants, as nearly 84% of the latter had increased susceptibility to this antibiotic. Increased sensitivity to meropenem was also the most common phenotypes amongst cetylpyridinium chloride-sensitive mutants, and 56% of the latter had decreased MICs to this antibiotic. It is interesting however that all of the cetylpyridinium chloride-resistant mutants were also sensitive to meropenem. Meropenem and azithromycin sensitivity was the most common phenotype among mutants susceptible to triclosan and *ortho*-phthalaldehyde. Over 72% of all *ortho*-phthalaldehyde-susceptible mutants had increased sensitivity to meropenem and nearly 55% were sensitive to azithromycin. Identical results were obtained with triclosan-sensitive mutants, with the addition that 55% of these mutants had also increased sensitivity to piperacillin.

Table 3.12. Agar MICs for wild type *S. marcescens* Db11 and biocide mutants.

	CHX MIC (µg/ml) ± SD	CPC MIC (µg/ml) ± SD	TRI MIC (µg/ml) ± SD	OPA MIC (µg/ml) ± SD	Biocide
***S. marcescens* Db11**	18 ± (2.83)	100 ± (28.3)	3900 ± (141)	3000 ± (283)	
***E. coli* NCTC 1048**	4 ± (0)	12 ± (28.3)	0.1 ± (0)	3000 ± (283)	
Susceptible phenotype					
10-E7	18 ± (2.83)	70 ± (14.1)	3900 ± (141)	2800 ± (283)	CPC, OPA
9-D5	14 ± (2.83)	60 ± (14.1)	3900 ± (141)	3000 ± (283)	CHX, CPC
3-F2	18 ± (2.83)	95 ± (7.07)	3900 ± (141)	2800 ± (283)	CPC, OPA
9-D10*	18 ± (2.83)	75 ± (7.07)	3900 ± (141)	3000 ± (283)	CPC
11-B8	18 ± (2.83)	100 ± (28.3)	3900 ± (141)	2700 ± (424)	OPA
12-F6	15 ± (1.41)	100 ± (28.3)	3200 ± (283)	2700 ± (141)	CHX, TRI, OPA
7-C10	18 ± (2.83)	100 ± (28.3)	3200 ± (566)	2600 ± (283)	TRI, OPA
18-A3	15 ± (1.41)	85 ± (7.07)	3900 ± (141)	3000 ± (283)	CHX, CPC
22-D5	18 ± (2.83)	80 ± (14.1)	3900 ± (141)	2800 ± (283)	CPC, OPA
N6-B2	18 ± (2.83)	75 ± (7.07)	2800 ± (283)	2600 ± (141)	CPC, TRI. OPA
N5-B6	18 ± (2.83)	90 ± (14.1)	3300 ± (141)	3000 ± (283)	CPC, TRI
N5-D9	18 ± (2.83)	100 ± (28.3)	3000 ± (283)	3000 ± (283)	TRI
N4-F6	16 ± (2.83)	80 ± (14.1)	3900 ± (141)	3000 ± (283)	CHX, CPC
N2-B3	15 ± (1.41)	85 ± (7.07)	3600 ± (141)	2600 ± (283)	CHX, CPC, TRI, OPA
N2-A8	16 ± (2.83)	90 ± (14.1)	3900 ± (141)	3000 ± (283)	CHX, CPC
N5-G1	17 ± (1.41)	90 ± (14.1)	3900 ± (141)	3000 ± (283)	CHX, CPC
N2-F1	16 ± (1.41)	100 ± (28.3)	3900 ± (141)	3000 ± (283)	CHX
Resistant phenotype					
19-D3	31 ± (7.07)	160 ± (28.3)	3900 ± (141)	3100 ± (283)	CHX, CPC, OPA
8-C7	24 ± (2.83)	100 ± (28.3)	4100 ± (141)	3000 ± (283)	CHX, TRI
27-B8*	18 ± (2.83)	100 ± (28.3)	4000 ± (283)	3200 ± (283)	TRI, OPA
Mixed phenotype					
10-B6	18 ± (2.83)	80 ± (14.1)	3900 ± (141)	3200 ± (283)	CPC^s, OPA^r
3-A4	30 ± (5.66)	60 ± (14.1)	3300 ± (283)	3000 ± (283)	CHX^r, CPC^s, TRI^s
N5-B5	18 ± (2.83)	140 ± (28.3)	3600 ± (283)	2700 ± (283)	CPC^r, TRI^s, OPA^s
N5-G6	28 ± (2.83)	100 ± (28.3)	3400 ± (566)	2600 ± (283)	CHX^r, TRI^s, OPA^s
N2-F3	28 ± (2.83)	100 ± (28.3)	3300 ± (141)	2800 ± (283)	CHX^r, TRI^s, OPA^s
N3-B8	26 ± (2.83)	200 ± (28.3)	3300 ± (424)	3000 ± (283)	CHX^r, CPC^r, TRI^s
Controls					
N3-C1*	18 ± (2.83)	100 ± (28.3)	3900 ± (141)	3000 ± (283)	
N1-C5	18 ± (2.83)	100 ± (28.3)	3900 ± (141)	3000 ± (283)	

SD; standard deviation, TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate. r, resistant, s, susceptible.
Increased resistance is highlighted in blue and increased sensitivity in red.
* Mutants not sequenced

Table 3.13. Biocide MIC ratios for the *S. marcescens* Db11 mutants.

	CHX		CPC		TRI		OPA		Phenotype
	MAX	MIN	MAX	MIN	MAX	MIN	MAX	MIN	
Susceptible									
10-E7	1.00	1.00	0.66	0.66	1.00	1.00	0.93	0.92	CPC, OPA
9-D5	0.80	0.75	0.58	0.55	1.00	1.00	1.00	1.00	CHX, CPC
3-F2	1.00	1.00	0.83	1.00	1.00	1.00	0.93	0.92	CPC, OPA
9-D10	1.00	1.00	0.66	0.77	1.00	1.00	1.00	1.00	CPC
11-B8	1.00	1.00	1.00	1.00	1.00	1.00	0.93	0.85	OPA
12-F6	0.80	0.87	1.00	1.00	0.85	0.78	0.87	0.92	CHX, TRI, OPA
7-C10	1.00	1.00	1.00	1.00	0.90	0.73	0.87	0.85	TRI, OPA
18-A3	0.80	0.87	0.75	0.88	1.00	1.00	1.00	1.00	CHX, CPC
22-D5	1.00	1.00	0.75	0.77	1.00	1.00	0.93	0.92	CPC, OPA
N6-B2	1.00	1.00	0.66	0.77	0.75	0.68	0.87	0.92	CPC, TRI. OPA
N5-B6	1.00	1.00	0.83	0.88	0.85	0.84	1.00	1.00	CPC, TRI
N5-D9	1.00	1.00	1.00	1.00	0.80	0.73	1.00	1.00	TRI
N4-F6	0.90	0.87	0.75	0.77	1.00	1.00	1.00	1.00	CHX, CPC
N2-B3	0.80	0.87	0.75	0.88	0.92	0.92	0.85	0.87	CHX, CPC, TRI, OPA
N2-A8	0.90	0.87	0.83	0.88	1.00	1.00	1.00	1.00	CHX, CPC
N5-G1	0.90	1.00	0.83	0.88	1.00	1.00	1.00	1.00	CHX, CPC
N2-F1	0.90	0.87	1.00	1.00	1.00	1.00	1.00	1.00	CHX
Resistant									
19-D3	1.80	1.62	1.50	1.55	1.00	1.00	1.03	1.03	CHX, CPC, OPA
8-C7	1.30	1.37	1.00	1.00	1.05	1.05	1.00	1.00	CHX, TRI
27-B8	1.00	1.00	1.00	1.00	1.05	1.00	1.06	1.07	TRI, OPA
Mixed									
10-B6	1.00	1.00	0.75	0.77	1.00	1.00	1.06	1.07	CPC^s, OPA^r
3-A4	1.70	1.62	0.58	0.55	0.87	0.81	1.00	1.00	CHX^r, CPC^s, TRI^s
N5-B5	1.00	1.00	1.33	1.33	0.95	0.89	0.87	0.85	CPC^r, TRI^s, OPA^s
N5-G6	1.50	1.62	1.00	1.00	0.95	0.78	1.00	1.00	CHX^r, TRI^s, OPA^s
N2-F3	1.50	1.62	1.00	1.00	0.85	0.84	0.93	0.92	CHX^r, TRI^s, OPA^s
N3-B8	1.40	1.50	1.83	2.00	0.90	0.78	1.00	1.00	CHX^r, CPC^r, TRI^s
Controls									
N3-C1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
N1-C5	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	

TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate. MAX and MIN represent respectively ratios generated from the maximum and the minimum MIC values for the biocide. Ratios represent the mutant values over those of the wild type. r; resistant (blue), s; sensitive (red)

Table 3.14. Effect of cetylpyridium chloride on maximal growth optical density of *S. marcescens* Db11 and its mutants in broth.

Organism	Mean $OD_{630\,nm}$	SE	P-value
***S. marcescens* Db11**	1.4213	0.0159	
***E. coli* NCTC 1048**	0.2847	0.0127	0.000
S. marcescens mutants:			
Value not different from that of the wild type			
3-F2	1.2170	0.1480	0.303
N2-F3	1.4383	0.0671	0.828
7-C10	1.2370	0.1260	0.283
12-F6	1.2200	0.0640	0.093
N1-C5	1.3583	0.0149	0.319
N2-F1	0.7210	0.2970	0.143
N2-B3	0.7880	0.2800	0.152
27-B8	1.1017	0.0397	0.121
N5-G6	1.1480	0.0723	0.149
N5-B6	1.5053	0.0558	0.285
N5-D9	1.5410	0.0311	0.549
11-B8	1.1980	0.1460	0.269
N3-C1	1.3450	0.2930	0.900
N5-G1	1.1860	0.0544	0.053
Value lower than that of the wild type			
3-A4	1.1670	0.0538	0.045
10-B6	0.5920	0.0439	0.003
22-D5	0.9260	0.0338	0.006
N2-A8	0.8770	0.0342	0.005
9-D10	0.6410	0.1830	0.050
8-C7	0.9467	0.0721	0.023
10-E7	0.6040	0.0778	0.009
9-D5	0.4730	0.1260	0.017
18-A3	0.9327	0.0170	0.000
N6-B2	0.2293	0.0213	0.000
N4-F6	0.7673	0.0853	0.017
Value higher than that of the wild type			
N3-B8	1.6003	0.0317	0.037
N5-B5	1.4793	0.0367	0.040
19-D3	1.4947	0.0026	0.045

Mean $OD_{630\,nm}$; mean $OD_{630\,nm}$ value of culture of the organism grown overnight at 37°C in TSB containing 5 µg/ml cetylpyridium chloride, SE; standard error, P-value; P-value for the two-sample t-test. Mutants with mean $OD_{630\,nm}$ values that were statistically significantly different from that of the wild type *S. marcescens* Db11 at 95% confidence, are highlighted (red; value significantly smaller than that of the wild type, blue; value significantly higher than that of the wild type).

Table 3.15. Effect of chlorhexidine diacetate on maximal growth of *S. marcescens* Db11 and its mutants in broth.

Organism	Mean $OD_{630\ nm}$	SE	P-value
S. marcescens Db11	1.3800	0.0223	
E. coli NCTC 1048	0.2166	0.0028	0.000
S. marcescens mutants:			
Value not different from that of the wild type			
3-F2	1.3617	0.0357	0.692
N2-F3	1.4630	0.0276	0.101
7-C10	1.4033	0.0248	0.535
27-B8	1.3623	0.0639	0.818
8-C7	1.4197	0.0391	0.443
N1-C5	1.4083	0.0699	0.736
3-A4	1.4123	0.0347	0.490
10-B6	1.3380	0.0380	0.411
9-D10	1.3807	0.0729	0.994
11-B8	1.2420	0.1040	0.325
N2-B3	1.4847	0.0384	0.100
10-E7	1.2873	0.0288	0.084
N5-B5	1.3803	0.0632	0.996
22-D5	1.2043	0.0672	0.131
N5-B6	1.4513	0.0681	0.424
N5-D9	1.2783	0.0758	0.327
N3-C1	1.2988	0.0788	0.890
Value lower than that of the wild type			
N2-F1	1.1183	0.0398	0.011
N6-B2	0.6460	0.0707	0.010
12-F6	0.8003	0.0282	0.001
N5-G1	1.0387	0.0468	0.022
18-A3	1.0153	0.0423	0.005
N2-A8	1.0490	0.0333	0.004
N4-F6	0.8533	0.0290	0.001
9-D5	0.9423	0.0228	0.001
Value higher than that of the wild type			
19-D3	1.4990	0.0159	0.022
N5-G6	0.6177	0.0542	0.006
N3-B8	1.5550	0.0167	0.008

Mean $OD_{630\ nm}$; mean $OD_{630\ nm}$ value of culture of the organism grown overnight at 37°C in TSB containing 6 µg/ml chlorhexidine diacetate, SE; standard error, P-value; P-value for the two-sample t-test. Mutants with mean $OD_{630\ nm}$ values that were statistically significantly different from that of the wild type *S. marcescens* Db11 at 95% confidence, are highlighted (red; value significantly smaller than that of the wild type, blue; value significantly higher than that of the wild type).

Table 3.16. Suspension tests for the *S. marcescens* Db11 mutants.

Organism	Log_{10} reduction in cfu/ml			
	CPC*	CHX**	TRI***	OPA****
***S. marcescens* Db11 ± (SD)**	3.13 ± (0.48)	2.57 ± (0.22)	3.11 ± (0.14)	1.18 ± (0.10)
***E. coli* NCTC 1048**	8.97	8.97	8.97	5.57
S. marcescens mutants:				
Increased killing				
10-E7	4.20	2.77	2.87	2.16
9-D5	3.90	2.99	3.17	1.13
3-F2	3.46	2.64	2.92	2.60
9-D10	3.83	2.66	4.03	1.14
11-B8	1.80	2.45	2.77	1.95
12-F6	2.88	3.76	4.47	4.09
7-C10	2.86	3.53	5.04	1.26
22-D5	3.93	2.99	3.49	1.29
N6-B2	2.93	2.77	3.93	1.41
N2-F3	2.94	2.45	3.04	1.62
N5-D9	2.87	2.89	3.89	1.19
N2-F1	2.83	4.86	3.20	1.16
N2-B3	2.86	2.98	3.82	2.30
N5-G1	3.99	3.38	2.83	1.19
Reduced killing				
19-D3	2.56	1.96	2.92	1.03
8-C7	2.69	2.16	2.61	0.96
Mixed phenotype				
10-B6	3.90	2.88	2.72	0.79
3-A4	4.20	1.81	6.04	1.30
18-A3	3.61	3.10	2.54	1.03
27-B8	2.84	2.98	2.18	1.09
N5-B5	2.56	2.86	3.69	1.51
N5-G6	2.97	2.08	3.88	1.38
N3-B8	2.55	2.20	4.33	1.09
N4-F6	3.99	3.35	1.83	1.09
N2-A8	2.92	3.04	2.80	1.02
Phenotype similar to the wild type				
N5-B6	3.60	2.44	3.07	1.02
N1-C5	3.04	2.59	2.88	1.19
N3-C1	3.01	2.60	2.99	1.14

SD; standard deviation, TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate. Mutants with log_{10} reductions in cfu/ml that are statistically significantly different at 95% confidence from that of the wild type are highlighted (blue; value statistically significantly lower than that of the wild type, red; value statistically significantly higher than the wild type). *; exposed to 8 µg/ml cetylpyridinium chloride for 10 min, **; exposed to 20 µg/ml chlorhexidine diacetate for 10 min, ***; exposed to 4000 µg/ml triclosan for 5 min, ****; exposed to 50 µg/ml *ortho*-phthalaldehyde for 5 min.

Table 3.17. Potassium leakage (in ppm) from biocide-treated *S. marcescens* Db11 mutants.

Organism	TRI*	OPA**	CPC***	CHX****
***S. marcescens* Db11 ± (SD)**	0.8 ± (0.08)	0.5 ± (0.08)	0.1 ± (0.008)	1.07 ± (0.09)
***E. coli* NCTC 1048**	1.0156	0.7131	0.6652	0.9652
S. marcescens mutants:				
Increased leakage				
10-E7	0.8508	0.7234	0.7418	1.1332
9-D5	0.8090	0.4340	0.3173	1.5114
3-F2	0.8102	0.4511	0.2901	1.0778
9-D10	0.7984	0.4401	0.2034	1.0545
11-B8	0.7987	0.7239	0.1205	1.0731
12-F6	0.9639	0.6930	0.1069	1.7667
8-C7	2.4849	1.3118	0.7458	2.8085
7-C10	0.9698	0.5911	0.1091	1.0577
18-A3	0.7561	0.4820	1.7741	1.8325
22-D5	0.8100	1.8090	0.2228	1.0771
27-B8	0.7124	0.4448	1.1082	1.1453
N6-B2	0.9632	0.7811	0.5000	1.1313
N5-B6	0.9618	0.4900	0.3115	0.9840
N5-D9	0.9530	0.5105	0.9970	1.0565
N4-F6	1.5305	0.5182	0.1516	1.3529
N2-B3	0.8322	0.6848	0.0938	1.5542
N2-A8	0.8197	0.5162	0.1430	1.4046
N5-G1	0.8052	0.4703	0.1231	1.7423
Reduced leakage				
19-D3	0.6024	0.2459	0.0860	0.4348
N2-F1	0.8481	0.4701	0.0867	0.9378
Mixed phenotype				
10-B6	0.8072	0.3386	0.6789	1.0708
3-A4	1.0677	0.4480	0.8953	0.8160
N5-B5	1.5971	0.5929	0.0621	0.6439
N5-G6	0.9634	0.8930	0.0890	0.6520
N2-F3	1.6496	1.0723	0.1150	0.8150
N3-B8	1.1013	0.5013	0.0800	0.7128
Phenotype similar to the wild type				
N1-C5	0.7792	0.5053	0.0921	0.9314
N3-C1	0.8977	0.5697	0.1034	0.9876

SD; standard deviation, ppm; parts per million, TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate. Mutants with potassium concentrations that are statistically significantly different at 95% confidence from that of the wild type are highlighted (blue; value statistically significantly lower than that of the wild type, red; value statistically significantly higher than the wild type). *; exposed to 4000 µg/ml triclosan for 5 min, **; exposed to 50 µg/ml *ortho*-phthalaldehyde for 5 min, ***; exposed to 8 µg/ml cetylpyridinium chloride for 10 min, ****; exposed to 20 µg/ml chlorhexidine diacetate for 10 min.

Table 3.18. Antibiotics agar MICs for *S. marcescens* Db11 mutants.

Antibiotic	CL (μg/ml)	MP (μg/ml)	CI (μg/ml)	TM (μg/ml)	TS (μg/ml)	AZ (μg/ml)	PP (μg/ml)	TZ (μg/ml)	IP (μg/ml)	AK (μg/ml)
S. marcescens Db11 (± SD)	24 (8.00)	0.12 (0.05)	0.17 (0.04)	25.1 (8.55)	0.65 (0.13)	>256 (0)	2 (0)	0.71 (0.31)	0.59 (0.09)	8 (0)
E. coli NCTC 1048	4	0.050	0.016	2	0.094	4.66	2	0.5	0.25	2
S. marcescens mutants										
10-E7	16	0.047	0.125	32	0.38	128	2	0.5	0.38	12
9-D5	32	0.094	0.25	16	0.5	128	3	0.6	1.5	4
3-F2	32	0.064	0.19	48	0.5	>256	2	0.75	0.38	12
9-D10	32	0.079	0.25	24	0.75	>256	3	1.5	1.5	12
11-B8	32	0.094	0.19	32	1	192	3	0.75	0.38	12
12-F6	8	0.109	0.19	56	0.46	101.3	1.5	0.85	2.25	12
7-C10	16	0.064	0.19	32	0.5	64	2	0.75	1.5	12
18-A3	48	0.19	0.25	24	1.5	>256	4	2	2	8
22-D5	32	0.064	0.19	12	0.75	>256	2	0.5	0.38	8
N6-B2	16	0.064	0.125	16	0.75	96	1.5	0.5	0.38	4
N5-B6	16	0.047	0.25	32	1	>256	1	1	0.25	4
N5-D9	32	0.19	0.25	16	1	>256	3	1.5	1	6
N4-F6	32	0.19	0.25	12	1	>256	2	1	1	3
N2-B3	16	0.047	0.125	24	0.38	96	1.5	0.25	0.25	6

Table 3.18. Antibioitics agar MICs for *S. marcescens* Db11 mutants (continued).

Antibiotic	CL (µg/ml)	MP (µg/ml)	CI (µg/ml)	TM (µg/ml)	TS (µg/ml)	AZ (µg/ml)	PP (µg/ml)	TZ (µg/ml)	IP (µg/ml)	AK (µg/ml)
N2-A8	32	0.19	0.19	16	1	>256	2	1	1	6
N5-G1	96	0.19	0.19	16	1.5	>256	4	1	2	4
19-D3	32	0.064	0.19	72	1.5	>256	2	0.5	1	24
8-C7	24	0.064	0.19	48	1	>256	2	0.75	0.62	12
27-B8	48	0.19	0.25	32	1.5	>256	3	2	2	12
10-B6	24	0.094	0.094	32	0.75	>256	3	1.5	1.5	8
3-A4	32	0.064	0.25	32	0.75	192	3	0.75	6	12
N5-B5	16	0.047	0.19	32	0.5	>256	1.5	0.5	0.25	8
N5-G6	24	0.047	0.125	24	0.75	>256	1.5	0.38	0.25	6
N2-F3	32	0.047	0.094	48	0.19	>256	2	0.75	0.19	12
N3-B8	24	0.064	0.125	32	1	96	2	0.5	0.25	8
N2-F1	64	0.125	0.19	16	1.5	192	3	1	0.75	6
N1-C5	32	0.064	0.19	32	0.75	>256	2	0.75	0.38	8
N3-C1	32	0.064	0.19	32	0.75	>256	2	0.75	0.25	8

CL; chloramphenicol, MP; meropenem, CI; ciprofloxacin, TM; tobramycin, TS; trimethoprim/sulfamethoxazole (1/19), AZ; azithromycin, PP; piperacillin, TZ; ceftazidime, IP; imipenem, AK; amikacin, SD; standard deviation.
Statistically significant increases in resistance (blue) or susceptibility (red) are highlighted.

3.4.13 Determination of the disrupted genes in the *S. marcescens* Db11 mutants

The site of insertion of the mini-Tn*5* transposon into the genome of the *S. marcescens* Db11 mutants was determined by sequencing the product of the two steps PCR reaction described in section 3.3.12. The PCR reaction amplified the transposon-chromosome junctions in each mutant. This site was sequenced as described in section 3.3.12. The resulting sequence was used in conjunction with bioinformatic analysis to determine the exact location of the transposon insert and the disrupted gene affected. Artemis (929) program along with BLAST homology searches against the GeneBank (www.ncbi.nlm.nih.gov) were used to annotate the disrupted gene and its surrounding DNA in the *S. marcescens* Db11 mutants (section 3.3.12). Out of the 26 biocide mutants and the two control mutants only three were not able to be sequenced. The transposon insertions in the sequenced mutants were located in 14 genes coding for putative proteins with varying functions (Table 3.19). Transposon insertions mapping into the same genes were also observed; one putative gene had transposon insertion in nine of the mutants sequenced. The transposon-disrupted putative genes in the sequenced mutants were as follows.

3.4.13.1 Putative carbamoyl-phosphate synthase large subunit gene (*carB*), mutant 8-C7.

The transposon insertion in mutant 8-C7 was shown to be in an ORF coding for a 1075 amino acids long putative protein. The latter had a carbamoyl-phosphate synthase large subunit conserved domain, and showed high homology with the CarB from *S. proteamaculans*. On agar, mutant 8-C7 demonstrated resistance to both triclosan and chlorhexidine diacetate (Table 3.12). However, resistance to the latter biocide was not apparent in broth (Table 3.15). Suspension tests showed that triclosan, *ortho*-phthalaldehyde and chlorhexidine diacetate were all more effective at killing the mutant compared to the wild type (Table 3.16). These results were confirmed by those from the potassium leakage experiment, where the mutant was shown to leak larger amounts of potassium compared to the wild type when subjected to the above biocides (Table 3.17).

Mutant 8-C7 is therefore more resistant to inhibition of growth by triclosan and chlorhexidine diacetate, however it is more sensitive to the killing effect of these two biocides. Antibiotic susceptibility profiles for the 8-C7 mutant indicated that it showed increase resistance to the two aminoglycosides amikacin and tobramycin but was more susceptible than the wild type to meropenem (Table 3.18).

Upstream of the putative *carB* gene was a 395 amino acid long putative carbamoyl-phosphate synthase small subunit encoding gene (*carA)* (Figure 3.12). The protein had a carbamoyl-phosphate synthase small subunit conserved domain, involved in both amino acid and nucleotide transport and metabolism in the cell. The protein had high homology with the CarA of *S. proteamaculans* and a number of other *Enterobacteriaceae* species. Adjacent to the putative *carA*, was a putative dihydrodipicolinate reductase (*dapB*) gene (Figure 3.12). The latter coded for a 284 amino acid long protein with high homology to the DapB from *S. proteamaculans* involved in amino acid transport and metabolism in the cell. Downstream from the putative *carB* gene was an ORF coding for a 216 amino acid long protein (Figure 3.12). It had a glutathione-regulated potassium-efflux system ancillary protein KefF conserved domain and showed high homology with the product of the *yabF* gene from *Shigella boydii*. All of the ORFs described above were transcribed in the same direction, including the one disrupted by transposon insertion (Figure 3.12). A summary of information about the gene disrupted by transposon insertion in mutant 8-C7 and its surroundings is shown in Table 3.30.

3.4.13.2 Putative DeoR family transcriptional regulator gene, mutants 10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3, and N2-F3.

The transposon insertions in nine mutants (10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3, and N2-F3) were all located in a single ORF encoding a 266 amino acid long protein (Figure 3.21). The protein had a putative transcriptional regulator of the DeoR family conserved domain and showed high homology to the DeoR from species of *Serratia* and *Burkholderia*. All but three of the above mutants showed increased sensitivity to cetylpyridinium chloride, and six of the mutants were sensitive to *ortho-*

phthalaldehyde. Mutants 3-A4, N2-B3, 7-C10, and N2-F3 were also sensitive to triclosan, and mutants 18-A3, 9-D5 and N2-B3 all showed increased sensitivity to chlorhexidine diacetate. Interestingly, mutants 3-A4 and N2-F3 expressed a resistant phenotype to the latter biocide (Table 3.12). Results of the killing tests (Table 3.16) and the potassium leakage experiments (Table 3.17) largely supported the mutants' above phenotypes. These tests showed that mutants sensitive to a particular biocide were more readily killed by that biocide and leaked larger amounts of potassium when exposed to the same biocide than did the wild type. On the other hand, mutants with resistant phenotypes, such as 3-A4 which showed increased resistance to chlorhexidine diacetate, leaked less potassium and suffered less killing when exposed to the biocide compared to the wild type. Antibiotic susceptibility profiles for the mutants (Table 3.18) showed that nearly 67% of these had increased sensitivity to azithromycin and a similar percentage to meropenem. Amikacin resistance was seen in 56% of the mutants. Mutant 18-A3 was of particular interest as it developed resistance to 8 out of the 10 antibiotics tested (Table 3.18).

Upstream of the putative DeoR regulator, was an ORF coding for a 226 amino acid long protein (Figure 3.21). The BLAST search did not show any conserved domain in the protein, however the latter showed high homology to an integral membrane protein from *Bradyrhizobium sp.* BTAi1. The protein is predicted to be a divalent heavy-metal cation transporter involved in inorganic ion transport and metabolism. Adjacent to this, laid an ORF coding for a 213 amino acid long protein. The latter did not show any conserved domains, however it showed homology with the hypothetical protein Spro_0996 from *S. proteamaculans* and the N-terminal of the heat shock protein DnaJ of *P. syringae*. Both of the above ORFs were encoded in direction opposite to that of the putative DeoR regulator gene (Figure 3.21). Downstream of the latter were located four ORFs (Figure 3.21). The first coded a short 148 amino acid long protein with a glyoxalase/bleomycin resistance conserved domain belonging to the dioxygenase protein superfamily. Adjacent to it and encoded in the opposite direction was an ORF coding for a 331 amino acid protein that showed high homology to a LysR family transcriptional regulator. The protein had a LysR substrate binding domain similar to that of periplasmic binding

proteins. Adjacent to the putative LysR regulator and encoded in the opposite direction were two ORFs. The first coded a 300 amino acid long protein with a pirin conserved domain. The protein showed homology to pirin-like proteins from species of *Serratia, Burkholderia*, and *Pseudomonas*. The second ORF coded for a 256 amino acid long protein with high homology to the product of the *ycaC* gene of *E. coli*. The protein had a YcaC related amidohydrolases conserved domain related to the isochorismatase family of hydrolase enzymes. A summary of information about the gene disrupted by transposon insertion in mutants 10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3, and N2-F3 and its surroundings is shown in Table 3.20.

3.4.13.3 Putative succinate dehydrogenase hydrophobic membrane anchor protein gene (*sdhD*), mutant 22-D5.

The transposon insertion in mutant 22-D5 was shown to be in a putative succinate dehydrogenase hydrophobic membrane anchor protein gene (*sdhD*) (Figure 3.20). On agar, the mutant demonstrated increase susceptibility to *ortho*-phthalaldehyde and cetylpyridinium chloride (Table 3.12). Sensitivity of mutant 22-D5 to cetylpyridinium chloride was also confirmed in broth (Table 3.14). Compared with the wild type, the mutant was more readily killed by triclosan, cetylpyridinium chloride and chlorhexidine diacetate, but not *ortho*-phthalaldehyde (Table 3.16). It was also shown that when subjected to *ortho*-phthalaldehyde or cetylpyridinium chloride, the mutant leaked larger amounts of potassium compared to the wild type (Table 3.17). Mutant 22-D5 showed increased sensitivity to both meropenem and tobramycin (Table 3.18).

The protein encoded by the putative *sdhD* gene was 176 amino acids long with a succinate dehydrogenase D (SdhD) subunit conserved domain. The protein belongs to the succinate, quinone oxidoreductase type C subfamily involved in electron transport and energy production and conversion in the cell. The protein showed high homology with the SdhD protein from *Yersinia* species. Upstream of the putative SdhD gene, and encoded in the opposite direction, laid a 1293 bp ORF coding for a 430 amino acids long putative protein (Figure 3.20). The protein had a type II citrate synthase conserved

domain known to be involved in energy production and conversion, and showed high homology with the GltA from *Serratia*, *Yersinia*, *Salmonella*, *Escherichia* and other *Enterobacteriaceae*.

Downstream from the putative SdhD gene, were three ORFs all encoded in the same direction as the putative *sdhD* gene (Figure 3.20). The first coded for a 588 amino acids long protein with high homology to the succinate dehydrogenase catalytic subunit SdhA of many *Enterobacteriaceae*, including species of *Serratia*, *Yersinia*, *Salmonella*, *Shigella* and *Escherichia*. The protein had a succinate dehydrogenase flavoprotein subunit conserved domain known to be involved in the energy production and conversion in the cell. The second ORF encoded a 261 amino acids long protein also thought to be involved in energy production and conversion. The protein had a putative succinate dehydrogenase iron-sulfur subunit conserved domain and showed high homology with the succinate dehydrogenase and fumarate reductase iron-sulfur protein from *Serratia* and the succinate dehydrogenase iron-sulfur catalytic subunit (SdhB) of many other *Enterobacteriaceae*. The final ORF coded for a larger, 938 amino acids long protein, with high homology to the 2-oxoglutarate dehydrogenase E1 component (SucA) from a number of *Enterobacteriaceae*. Dehydrogenase (E1) component conserved domain of the protein is involved in the process of energy production and conversion in the cell.

All of the ORFs described, including that which has the transposon insertion, had a G+C content within a close range of the mean 59.5% of the *S. marcescens* Db11 genome (Table 3.21). The putative *sdhD* gene disrupted by the transposon insertion in mutant 22-D5 appears to be part of a putative operon involved in energy processes in the cell, which includes at least three genes (*sdhA*, *sdhB*, and *sdhD*). These genes are known to encode for components of a four member succinate dehydrogenase enzyme complex that forms a trimeric complex. The SdhA/B are the catalytic subcomplex and can exhibit succinate dehydrogenase activity in the cell in the absence of SdhC/D which are the membrane components and form cytochrome b556 (188, 1044). A summary of information about the gene disrupted by transposon insertion in mutant 22-D5 and its surroundings is shown in Table 3.21.

3.4.13.4 Putative cell envelope biogenesis operon genes, mutants N4-F6, N2-A8, N2-F1, N5-G1, and N5-D9.

Five biocide mutants mapped to the same cluster of genes part of a putative operon consisting of at least four ORFs. The first of these ORFs had a low 45.5% G+C content and coded for a 445 amino acid long putative protein. BLAST searches showed that the protein had a methyltransferase conserved domain, and showed homology with the methyltransferase type 11, product of the *wbbD* gene, from species of *Klebsiella, Burkholderia*, and *Pseudomonas*. Adjacent to the putative *wbbD* gene was another low G+C content ORF (47.3%) coding for a large, 1219 amino acid long, putative protein. The protein had a glycosyltransferase conserved domain, known to be involved in the biosynthesis of disaccharides, oligosaccharides and polysaccharides. The protein also showed homology with the mannosyltransferase A, product of the *wbdA* gene from *Klebsiella* and *E. coli* and with group 1 glycosyltransferase from species of *Pseudomonas*, *Prochlorococcus* and *Methylobacterium*. Both proteins are known to be involved in the cell envelope's lipopolysaccharides biogenesis. The third ORF had a 42.2% G+C content and coded for a 381 amino acid putative protein which also had a glycosyltransferase group 1 conserved domain. The protein showed homology to the WbdB (mannosyltransferase B) from *Klebsiella* and *E. coli*, and glycosyltransferase (WbpY) from *Pseudomonas* species. The final ORF also had a low G+C content (44.5%) and coded for a 378 amino acid long putative protein with a glycosyltransferase group 1 conserved domain. It showed homology with the glycosyltransferase (WbpZ) from *Pseudomonas* species and the WbcW protein from *Yersinia enterocolitica*. The putative *wbbD*, *wbdA*, *wbdB*, and *wbpZ* genes appear to be part of a putative outer membrane lipopolysaccharides biogenesis operon.

Upstream from the above putative operon, and encoded in the same direction, was a 1320 bp ORF, with a low 44.2% G+C content, coding for a 439 amino acid long putative protein. The latter had a KpsT/Wzt ABC transporter subfamily conserved domain, involved in extracellular polysaccharide export. The protein showed high homology with the product of the *wzt* gene from *E. coli* and *Pseudomonas* species. Downstream from the

putative operon, and also encoded in the same direction, laid an ORF with a 57.4% G+C content coding for a 468 amino acid long putative protein. The latter showed high homology with the 6-phosphogluconate dehydrogenase coded by the *gnd* gene of a number of *Enterobacteriaceae,* including species of *Serratia*, *Yersinia*, and *Klebsiella.* The protein conserved domain (6-phosphogluconate dehydrogenase) is involved in carbohydrate transport and metabolism and act by catalyzing the formation of D-ribulose 5-phosphate from 6-phospho-D-gluconate in the cell. The low G+C content of all of the above ORFs compared to that of the *S. marcescens* Db11 genome (Table 3.22, Figure 3.15), suggests that both the putative biogenesis operon and its two surrounding genes may be located on an acquired piece of DNA not native to the organism.

Transposon insertions in 4 mutants (N4-F6, N2-A8, N2-F1 and N5-G1) were located within the putative *wbdA* gene (Figure 3.15). On agar, all these mutants showed increase susceptibility to chlorhexidine diacetate and three out of the four (N4-F6, N2-A8, and N5-G1) also showed increase sensitivity to cetylpyridinium chloride (Table 3.12). Broth MICs (Table 3.14) confirmed the results from the agar experiments. Suspension tests (Table 3.16) showed that all four mutants were more readily killed by chlorhexidine diacetate than was the wild type, and that two of them (N4-F6 and N5-G1) were more susceptible to the killing effect of cetylpyridinium chloride compared with the wild type. With the exception of mutant N2-F1, all mutants leaked more potassium than the wild type when exposed to chlorhexidine diacetate or cetylpyridinium chloride. These results strongly suggested that disruption of the *wbdA* gene lead to increase sensitivity to chlorhexidine diacetate and cetylpyridinium chloride both in terms of inhibition of growth and killing effect. Antibiotic susceptibility profiles for the above four mutants (Table 3.18) showed that all had increased sensitivity to the two aminoglycosides, amikacin and tobramycin, and that three of them showed increased resistance to meropenem.

A fifth mutant (N5-D9) had a transposon insertion in the putative *wbpZ* gene (Figure 3.15). The mutant showed increased sensitivity to triclosan on agar, and was shown to be more readily killed and leaked greater amounts of potassium when exposed to the same

biocide compared with the wild type. Evidence of increase sensitivity to the killing effect of chlorhexidine diacetate and cetylpyridinium chloride was also observed (Tables 3.16). Mutant N5-D9, similar to the above four putative *wbdA* mutants also showed increased sensitivity to amikacin and tobramycin, and increased resistance to meropenem. It also was more resistant than the wild type to ciprofloxacin, piperacillin, and ceftazidime (Table 3.18). A summary of information about the genes disrupted by transposon insertion in mutants N4-F6, N2-A8, N5-G1, N2-F1 and N5-D9 and their surroundings is shown in Table 3.22.

3.4.13.5 Putative outer membrane protein A gene (*ompA*), mutant 12-F6.

The transposon insertion in mutant 12-F6 was mapped to an ORF that coded for a 371 amino acid long putative protein with high homology to the outer membrane protein A (OmpA) from a number of *Enterobacteriaceae*, including species of *Serratia*, *Yersinia*, *Shigella* and *Klebsiella*. The OmpA-like transmembrane domain of the protein is a member of the outer membrane beta-barrel protein superfamily clan involved in cell wall and membrane biogenesis. OmpA is a porin, involved in diffusion of non-specific small solutes across the outer membrane. On agar, mutant 12-F6 showed increased susceptibility to triclosan, *ortho*-phthalaldehyde, and chlorhexidine diacetate (Table 3.12). Increased sensitivity to the latter biocide was also confirmed in broth (Table 3.15). The mutant was more susceptible to the killing effect of the three biocides (Table 3.16), and leaked greater amounts of potassium when exposed to these biocides compared with the wild type (Table 3.17). Mutant 12-F6 showed increased sensitivity to chloramphenicol, azithromycin, and piperacillin. It was however also more resistant than the wild type to two aminoglycosides, amikacin and tobramycin, and to imipenem (Table 3.18).

Downstream from the putative *ompA* and encoded in the opposite direction was an ORF coding for a small, 161 amino acid long, putative protein. The latter had a hypothetical protein multidomain of unknown function and showed high homology to a number of conserved hypothetical proteins from a number of *Enterobacteriaceae* as well as the

putative cytoplasmic protein YcbG from *E. coli*. Adjacent to the above ORF and encoded in the opposite direction was an ORF which coded for a 594 amino acid long putative protein. Blast searches revealed that the protein had high homology with a number of ATP-dependent proteases from *Enterobacteriaceae* especially the Lon protease of *Serratia* and *Yersinia*. The protein conserved domain (ATP-dependent protease) is known to be involved in posttranslational modification and protein turnover and acts as chaperone in the cell.

Upstream from the putative *ompA*, was a low G+C content ORF coding for a small 178 amino acid long putative protein. It had a SOS-response cell division inhibitor conserved domain involved in cell cycle control and the control of mitosis and meiosis by blocking the FtsZ ring (77, 210) formation during cell division and chromosome partitioning. The protein showed high homology with the cell division inhibitor SulA of *Serratia*. Adjacent to the putative *sulA*, and encoded in the opposite direction, was an ORF coding for a 228 amino acid long putative protein with a Tfox conserved domain. Tfox is known to be a regulator of competence-specific genes. Downstream from the above ORF and encoded in the same direction as the putative *ompA*, was an ORF coding for a large 730 amino acid long putative protein. The protein had a bacterial membrane protein of unknown function conserved domain. This family consists of several putative bacterial membrane proteins of unknown function. A number of genes coding for such proteins have been annotated as putative efflux transporters such as that of *Salmonella* and *Yersinia,* whereas the gene from *E. coli* has been annotated as *yccS*. The protein showed high homology with hypothetical membrane protein of *Serratia*, and the putative efflux transporter (PET) family from *Yersinia* and *Salmonella*. A summary of information about the gene disrupted by transposon insertion in mutant 12-F6 and its surroundings is shown in Table 2.23.

3.4.13.6 Putative ribonucleotide-diphosphate reductase alpha subunit gene (*nrdA*), mutant N5-B6.

The transposon insertion in mutant N5-B6 was traced to an ORF coding for a 780 amino acid long putative protein with a ribonucleotide reductases conserved domain (Figure 3.22). These proteins are found in all organisms and provide the only mechanism by which nucleotides are converted to deoxynucleotides. They catalyze the reductive synthesis of deoxyribonucleotides from their corresponding ribonucleotides, therefore providing the precursors necessary for DNA synthesis. The encoded protein showed high homology with product of the *nrdA* gene (ribonucleotide-diphosphate reductase α subunit) from *Shigella*, and *Yersinia*. The N5-B6 mutant showed increased sensitivity to triclosan and cetylpyridinium chloride on agar (Table 3.12). Although suspension tests did not show any increase in the killing effect of the two biocides on the mutant, it leaked greater amounts of potassium than the wild type when exposed to these two biocides (Table 3.17). Antibiotic susceptibility profiles for the N5-B6 mutant (Table 3.18) showed that it had increased sensitivity to the two carbapenems, meropenem and imipenem, as well as to piperacillin, and amikacin. The mutant was however more resistant than the wild type to ciprofloxacin.

Downstream from the putative *nrdA* gene was an ORF coding for a 384 amino acid long putative protein with high homology to the ribonucleotide-diphosphate reductase β subunit (NrdB) from a number of *Enterobacteriaceae*. The protein's conserved domain is part of the superfamily of ferritin-like diiron-carboxylate proteins and is involved in nucleotide transport and metabolism in the cell. It appears that the *nrdA* and *nrdB* genes are part of a putative operon whos products result in the formation of a multimeric complex, NrdA/NrdB. Upstream of the putative *nrdA* gene laid two ORFs encoded in opposite directions and coding for a 280 and a 885 amino acid long putative proteins respectively. The first protein had a methyltransferase (3-demethylubiquinone-9 3-methyltransferase) conserved domain, involved in ubiquinone biosynthesis and coenzyme metabolism. The protein showed high homology with 3-demethylubiquinone-9 3-methyltransferase proteins, products of the *pufX* gene from *Yersinia* and the *ubiG* genes

of *Erwinia*, *Escherichia*, *Klebsiella* and other *Enterobacteriaceae.* The second ORF coded for a larger putative protein with a DNA gyrase subunit A conserved domain. DNA gyrase is involved in negatively supercoiling closed circular double-stranded DNA and is an important protein in DNA replication, recombination and repair in the cell. BLAST searches showed that the protein was identical to the GyrA from *S. marcescens.* A summary of information about the gene disrupted by transposon insertion in mutant N5-B6 and its surroundings is shown in Table 3.24.

3.4.13.7 Putative nucleoid-associated protein gene (*ndpA*), mutant N3-B8.

Agar MICs demonstrated that mutant N3-B8 had increased sensitivity to triclosan. In contrast it increased resistance to cetylpyridinium chloride and chlorhexidine diacetate (Table 3.12). Increase resistance to the two biocides was also confirmed in broth (Tables 3.14 and 3.15). Triclosan was shown to kill the mutant more effectively than it did the wild type (Table 3.16), and that the mutant leaked larger amounts of potassium when exposed to this biocide than the wild type (Table 3.17). The mutant was killed less effectively by cetylpyridinium chloride and chlorhexidine diacetate and released smaller amounts of potassium when exposed to these two biocides compared to the wild type (Tables 3.16 and 3.17). These results demonstrated that triclosan was more effective at killing and inhibiting the growth of mutant N3-B8 compared with the wild type. However, the mutant was more resistant to the killing and the growth inhibitory effects of cetylpyridinium chloride and chlorhexidine diacetate than the wild type. Antibiotic susceptibility profiles for the N3-B8 mutant (Table 3.18) showed that it had increased sensitivity to the two carbapenems, meropenem and imipenem, as well as to ciprofloxacin and azithromycin.

The ORF disrupted by transposon insertion in mutant N3-B8 coded for a 341 amino acid long putative protein which had a nucleoid-associated protein NdpA conserved domain. The protein showed high homology to the 37-kDa nucleoid-associated protein from *S. proteamaculans* and the NdpA from other *Enterobacteriaceae* including *Erwinia*, *Escherichia*, *Klebsiella, Salmonella* and *Yersinia.* Upstream of the putative *ndpA* were

three ORFs, all encoded in the opposite orientation to the putative *ndpA* (Figure 3.22). The first ORF coded for a 594 amino acid long putative protein with high homology to the *Serratia* sulfatase and to the product of the *yejM* gene from a number of *Enterobacteriaceae* including, *Escherichia*, *Salmonella* and *Shigella.* The protein had both a predicted hydrolase of alkaline phosphatase superfamily conserved multi-domain, and a phosphodiest domain. The second ORF coded for a small 153 amino acid long putative protein with a conserved domain that belonged to a family of small uncharacterised proteins. In a DNA-binding protein from *Caulobacter vibrioides* this domain is found next to a DNA binding helix-turn-helix domain, suggesting that this is some kind of ligand binding domain. The third ORF coded for a 373 amino acid long putative protein which showed high homology to acyltransferase 3 from *Ralstonia, Anabaena,* and *Burkholderia* and to the putative acyltransferase transmembrane proteins from *Ralstonia* species. The protein's predicted acyltransferases conserved domain is involved in lipid metabolism. The noticeably low G+C content of this ORF (37.7%) compared to that of the *S. marcescens* Db11 DNA suggests that it could be related to its function rather than it being not native to this organism.

Downstream of the putative *ndpA* were four ORFs, two of which were encoded in the same direction as the putative *ndpA* and the other two were encoded in the opposite direction (Figure 3.22). The first ORF immediately adjacent to the putative *ndpA*, had a low G+C content (47.1%) and coded for a small 129 amino acid long putative protein with high homology to the 50S ribosomal subunit protein L25 (RplY) from a number of *Enterobacteriaceae.* The protein is involved in ribosomal proteins synthesis and modification and the process of translation. Next to the putative *rplY* was an ORF coding for 600 amino acid long putative protein. The protein had homology with proteins of the DNA or RNA helicases of superfamily II involved in transcription and DNA replication, recombination, and repair. The third ORF coded for a 267 amino acid long putative protein with homology to the ribosomal small subunit pseudouridine synthase A (RsuA) from *Escherichia* and *Yersinia,* and other 16S rRNA uridine-516 pseudouridylate synthase and related pseudouridylate synthases. Pseudouridine synthases catalyze the isomerization of specific uridines in an RNA molecule to pseudouridines (395). The

putative *rsuA* was adjacent to a fourth ORF coding for a 432 amino acid long putative protein. The latter showed high homology to a drug resistance transporter of the Bcr/CflA subfamily from *S. proteamaculans*, and the bicyclomycin resistance protein (Brc) from *Escherichia*, *Salmonella*, *Shigella* and *Erwinia*. The protein had a multidrug resistance protein D (EmrD) conserved domain, a protein belonging to the MFS pumps superfamily, and to the bicyclomycin/multidrug efflux system protein involved in sulfonamide (sulfathiazole) and bicyclomycin resistance (65). A summary of information about the gene disrupted by transposon insertion in mutant N3-B8 and its surroundings is shown in Table 3.25.

3.4.13.8 Putative pili operon gene, mutant N5-B5.

Mutant N5-B5 had a transposon insertion located within a low G+C content (48%) ORF that coded for a 249 amino acid long putative protein (Figure 3.14). The protein had a FimC, P pilus assembly protein, chaperone PapD conserved domain, which is involved in cell motility as well as intracellular trafficking and secretion. The protein showed high homology to the pili assembly chaperone of *S. proteamaculans* and the PapD protein of *E. coli*. Agar MICs showed that mutant N5-B5 was more sensitive to triclosan and *ortho*-phthalaldehyde than the wild type but had increased resistance towards cetylpyridinium chloride (Table 3.12). The latter phenotype was confirmed by broth experiments (Table 3.14). Similarly, suspension tests demonstrated that larger numbers of N5-B5 cells were killed upon exposed to triclosan or *ortho*-phthalaldehyde compared to wild type (Table 3.16). On the other hand, cetylpyridinium chloride exposed to the mutant had a lesser killing effect than that observed with the wild type (Table 3.16). Compared with the wild type, N5-B5 was also shown to leak greater amounts of potassium when exposed to triclosan but leaked less potassium when exposed to cetylpyridinium chloride (Table 3.17). These observations confirmed that mutant N5-B5 was more resistant to both the killing and inhibitory effects of cetylpyridinium chloride. In contrast, it was more sensitive to growth inhibition by *ortho*-phthalaldehyde and triclosan and to the killing effect of at least the latter. Mutant N5-B5 exhibited increased sensitivity to the two carbapenems, meropenem and imipenem, as well as to piperacillin.

Upstream of the transposon disrupted gene were located two ORFs. The first was immediately adjacent to the disrupted ORF, with a low G+C content (51.8%) and encoded in the same orientation. It coded for a 221 amino acid putative protein with high homology to the mannose sensitive type I fimbriae major structural subunit, product of the *safA* gene of *S. marcescens*, and to the P pilus assembly protein, pilin FimA from *E. coli*. The protein's FimA conserved domain, is a member of the bacterial adhesin superfamily clan involved in cell motility, secretion, and cell adhesion. The second ORF is encoded in the opposite direction of the putative *safA*, and coded for a 250 amino acid long putative protein with an inner membrane protein conserved domain. In BLAST searches the protein showed homology to the putative carrier/transport protein, product of the *yccA* gene from *Shigella* and *Escherichia*, to the hypothetical protein ECA1763 from *Erwinia*, and to the protein of unknown function UPF0005 from *Serratia*.

Immediately downstream of the disrupted gene was an ORF encoded in the same direction and coding for a large 834 amino acid long putative protein. The protein had homology with the FimD, P pilus assembly protein (porin PapC), conserved domain involved in cell motility, intracellular trafficking and secretion in the cell. It also had homology with the fimbrial usher protein conserved domain which is involved in biogenesis of gram negative bacterial pili. BLAST searches demonstrated that the protein was highly homologous to the PacC of *E. coli*, and to the fimbrial biogenesis outer membrane usher protein of *S. proteamaculans*. In Gram-negative bacteria the biogenesis of fimbriae (pili) requires a two-component assembly and transport system which is composed of a periplasmic chaperone and an outer membrane protein which has been termed a molecular "usher" (104). The location and function of the above ORF, the putative *safA* and the transposon disrupted gene suggested that they may be part of a putative pili operon in *S. marcescens* Db11.

Downstream of the putative pili operon were located three short, low G+C content, ORFs, all encoded in the same orientation (Figure 3.14). The first coded for a 374 amino acid long putative protein with no detected conserved domain and which showed

homology to the hypothetical proteins EcolE1_01003164 from *E. coli* and Spro_4156 from *S. proteamaculans*. This ORF was also the site of transposon insertion in the control mutant N1-C5. The second ORF coded for a shorter, 188 amino acid long, putative protein with low homology to the conserved domain of RelB, DNA-damage-inducible protein J, involved in DNA replication, recombination, and repair in the cell. The putative protein showed low homology to both the DNA-damage-inducible protein J from *S. boydii* and to the hypothetical protein Spro_4157 from *S. proteamaculans*. The final ORF coded for another short, 178 amino acid long, putative protein with a GerE, bacterial regulatory protein of the luxR family, conserved domain. This family is a member of the helix-turn-helix clan involved in sequence-specific DNA regulation. BLAST searches showed that the protein had low homology to the two-component transcriptional regulator of the LuxR family from *Rubrobacter xylanophilus.* The above three ORFs all had low G+C content (Table 3.26) and may have been acquired from a foreign source.

Adjacent to these three ORF and encoded in the opposite direction was another short ORF coding for a putative redox-sensitive transcriptional activator soxR. The putative 164 amino acid long protein had a helix-turn-helix transcriptional regulator SoxR conserved domain. In *E. coli*, this global regulator up-regulates gene expression of another transcription activator, SoxS, which directly stimulates the oxidative stress regulon genes (14). The *soxRS* response renders the bacterial cell resistant to superoxide-generating agents, macrophage-generated nitric oxide, organic solvents, and antibiotics (703, 743, 885). Finally downstream of the putative *soxR*, and encoded in the opposite direction, was an ORF coding for a 403 amino acid long putative protein with homology to the RND efflux membrane fusion protein precursor (MexH). The protein had a multidrug efflux system subunit MdtA conserved domain, known to be part of a tripartite efflux system composed of MdtA, MdtB and MdtC, which confers resistance against novobiocin and deoxycholate. A summary of information about the gene disrupted by transposon insertion in mutant N5-B5 and its surroundings is shown in Table 3.14.

3.4.13.9 Putative 6-phosphofructokinase gene (*pfkA*), mutant N6-B2.

The transposon insertion in the genome of mutant N6-B2 was located within an ORF coding for a 352 putative protein which had a phosphofructokinase conserved domain (Figure 3.18). The latter is a key regulatory enzyme in glycolysis, and catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-biphosphate. The putative protein showed high homology with the 6-phosphofructokinase of *Serratia* and the product of *pfkA* gene of *Escherichia* and a number of other bacterial species. Agar MIC tests indicated that mutant N6-B2 was more sensitive to triclosan, *ortho*-phthalaldehyde, and cetylpyridinium chloride than the wild type (Table 3.12). Increased sensitivity to the latter biocide was also confirmed in broth (Table 3.14). Similarly, suspension tests showed that the mutant was more effectively killed, and leaked larger amounts of potassium when exposed to the three above biocides than the wild type (Tables 3.16 and 3.17). These results indicated that the mutant was more sensitive to both the killing and the growth inhibitory effect of triclosan, *ortho*-phthalaldehyde, and cetylpyridinium chloride than the wild type. Antibiotic susceptibility profiles for mutant N6-B2 (Table 3.18) showed that it had increased sensitivity to 6 out of the 10 antibiotic tested. These were meropenem, ciprofloxacin, tobramycin, azithromycin, piperacillin and amikacin.

Downstream from the putative *pfkA* gene were two ORFs (Figure 3.18), the first of which was immediately adjacent to it and encoded in the same direction. This ORF coded for a 390 amino acid long putative protein with high homology to the sulfate transporter subunit of *Escherichia* and the periplasmic sulfate-binding protein of *Shigella*, both of which are products of the *sbp* gene. The highest homology was with the sulfate ABC transporter, periplasmic sulfate-binding protein of *S. proteamaculans*. The putative protein conserved domain is involved in inorganic ion transport and metabolism in the cell. The second ORF, encoded in the opposite orientation to the putative *pfkA*, coded for a 282 amino acid long putative protein. It had a triosephosphate isomerase conserved domain and showed high homology with triosephosphate isomerase proteins (TipA) from *Serratia*, *Salmonella*, *Yersinia*, *Erwinia* and other *Enterobacteriaceae*. These proteins are glycolytic enzymes that catalyze the interconversion of dihydroxyacetone phosphate and

D-glyceraldehyde-3-phosphate in the cell, and play an important role in several metabolic pathways, and are essential for efficient energy production (228).

Upstream of the putative *pfkA* were three ORFs (Figure 3.18), the first two of which were adjacent to each other and encoded in the same direction. The first of these ORFs coded for putative 339 amino acid long protein, with a FieF, ferrous iron efflux protein F conserved domain, a member of cation diffusion facilitator family involved in inorganic ion transport and metabolism. The protein is membrane-bound and induced by both zinc and iron, but does not induce resistance to zinc. It can transport zinc (II) in a proton-dependent manner and it is known to induce iron resistance (572, 1168). The putative protein coded by the above ORF showed high homology with the FieF of many *Enterobacteriaceae,* as well as the other cation diffusion facilitator family transporters such as the cadmium, zinc, and cobalt cation transporters.

The second ORF, coded for a smaller, 162 amino acid long, putative protein with homology to the P pilus assembly/Cpx signaling pathway, periplasmic inhibitor/zinc-resistance associated protein from *Serratia*, *Yersinia* and *Escherichia*, as well as a number of hypothetical proteins such as YPDSF_3831, YPO0075 and YPTB0071 from *Yersinia*, ESA_04124 from *Enterobacter* and Z5458 from *Escherichia*. The protein had a CpxP conserved domain, a periplasmic repressor of the Cpx envelope stress response pathway (214). Cpx repression by CpxP occurs via periplasmic interactions with CpxA (1174).

The third ORF was encoded in the opposite direction of the putative *pfkA*, and coded for a 234 amino acid long putative protein. The protein showed high homology with the CpxR transcriptional regulatory from a number of *Enterobacteriaceae,* and had a DNA-binding response regulator in two-component regulatory system with CpxA conserved domain. A summary of information about the gene disrupted by transposon insertion in mutant N6-B2 and its surroundings is shown in Table 3.27.

3.4.13.10 Putative outer membrane biogenesis operon gene (*wzzE*), mutants 19-D3 and 10-B6.

The ORF disrupted by transposon insertion in both mutant 19-D3 and 10-B6 coded for a 359 amino acid long putative protein. The protein had a lipopolysaccharide biosynthesis protein WzzE conserved domain and showed high homology to lipopolysaccharide biosynthesis proteins and to the enterobacterial common antigen polysaccharide chain length modulator from a number of *Enterobacteriaceae*, all products of the *wzzE* gene. These proteins are chain length determinant proteins involved in the cell envelope (outer membrane) biogenesis. Agar MICs and broth tests showed that mutant 19-D3 had increased resistance to cetylpyridinium chloride and chlorhexidine diacetate (Tables 3.14 and 3.15). The mutant was also resistant to *ortho*-phthalaldehyde on agar (Table 3.12). In addition, the mutant showed increased resistance to the killing effect of the above three biocides, leaking smaller amounts of potassium upon exposure to these compounds than the wild type (Tables 3.16 and 3.17). Mutant 10-B6 was similar to 19-D3 in that it showed increased resistance to both the killing and growth inhibitory effects of *ortho*-phthalaldehyde (Tables 3.16 and 3.12). However, the mutant showed increased sensitivity to cetylpyridinium chloride both in broth and on agar (Tables 3.12 and 3.14). The biocide was also more effective at killing the mutant than the wild type, leading to leakage of greater amount of potassium upon exposure to this biocide (Tables 3.16 and 3.17). Antibiotics susceptibility profiles for the two mutants (Table 3.18) showed that both mutants exhibited increased resistance to 3 out of the 10 antibiotic tested, and increased sensitivity to one antibiotic. However, there were no similarities between the antibiotic susceptibility profiles of the two mutants.

The putative *wzzE* gene was surrounded by four ORFs, two from each side and all encoded in the same direction as the disrupted gene (Figure 3.17). The first of the two ORFs upstream of the putative *wzzE* was immediately adjacent to the disrupted gene and coded for a 391 amino acid putative protein. The latter had a Rfe (UDP-*N*-acetylmuramyl pentapeptide phosphotransferase/UDP-*N*-acetylglucosamine-1-phosphate transferase) conserved domain involved in the cell envelope (outer membrane) biogenesis. The

protein had high homology to the product of the *rfe* gene from a number of bacterial species including the undecaprenyl-phosphate alpha-*N*-acetylglucosaminyl 1 phosphatetransferase involved in synthesis of enterobacterial common antigen in *E. coli*. The second ORF upstream of the putative *wzzE* coded for a 423 amino acid long putative Rho transcription termination factor. The putative protein conserved domain was an RNA-DNA helicase that actively releases nascent mRNAs from paused transcription complexes.

The ORF immediately downstream of the putative *wzzE* gene coded for a 398 amino acid long putative protein. The protein showed high homology to the UDP-N-acetyl glucosamine-2-epimerase, product of the *wecB* and *rffE* genes from a number of *Enterobacteriaceae*, including *Escherichia*, *Serratia*, *Klebsiella*, *Salmonella* and *Yersinia*. The protein conserved domain (WecB) is involved in cell envelope (outer membrane) biogenesis. Immediately adjacent to the putative *wecB* gene was an ORF coding for a 462 amino acid long putative protein. It had a WecC, UDP-*N*-acetyl-D-mannosamine dehydrogenase, conserved domain and showed high homology to the products of the *wecC* and *rffD* genes from a number of *Enterobacteriaceae*, including the UDP-glucose/GDP-mannose dehydrogenase from *Yersinia*, and the UDP-*N*-acetyl-D-mannosamine dehydrogenase from *Erwinia*. The putative *wzzE*, *rfe*, *rffE* and *rffD* genes appear to be part of a putative operon involved in the cell outer membrane biogenesis. A summary of information about the gene disrupted by transposon insertion in mutant 19-D3 and 10-B6, and its surroundings are shown in Table 3.28.

3.4.13.11 Putative chaperonin genes (*groES, groEL*), mutant N5-G6.

The site of transposon insertion in mutant N5-G6 was located within a region of the *S. marcescens* Db11 genome that consisted of five ORFs, four of which were encoded in the same direction (Figure 3.16). The first ORF, encoded in direction opposite to the other four, coded for a 493 amino acid long putative protein. The protein had an aspartase-like conserved domain, which contains a group of proteins similar to aspartase (L-aspartate ammonia-lyase) and fumarase class II enzymes, members of the Lyase class I family. These proteins catalyze the reversible deamination of aspartic acid. The protein showed

high homology with the AspA (aspartate ammonia-lyase) from a number of bacterial species, which catalyses the formation of fumarate from aspartate. The second ORF coded for a small, 165 amino acid long, putative FxsA cytoplasmic membrane protein. The latter is known to be a suppressor of F-plasmid exclusion of phage T7 in number of bacterial species including species of *Yersinia, Erwinia, Salmonella* and *Shigella.* The third ORF, also coded for a small putative protein (132 amino acid long), which had a GroES co-chaperonin conserved domain. The protein showed homology with the chaperonin Cpn10 from *Serratia* and *Enterobacter* as well as the co-chaperonin GroES from a number of other bacterial species. Adjacent to the putative *groES* gene, was an ORF coding for a 552 amino acid long putative protein, which showed high homology with the 60-kDa chaperonin, GroEL, from a number of *Enterobacteriaceae.*

Chaperonins are 'helper' molecules required for correct folding and subsequent assembly of some proteins. They are required for normal cell growth and are stress-induced, acting to stabilise or protect disassembled polypeptides under heat-shock conditions. In eubacterial, the 10-kDa chaperonin GroES, cooperates with GroEL to encapsulates non-native substrate proteins inside the cavity of the GroEL-ES complex and promotes folding by using energy derived from ATP hydrolysis (527, 618, 619). Adjacent to the putative *groEL* gene, laid an ORF coding for a 578 amino acid long putative protein. The protein had homology with the methyl-accepting chemotaxis protein and the methyl-accepting chemotaxis protein IV peptide sensor receptor, products of the *cheD* and *tap* genes respectively, in a number of bacterial species.

The site of the transposon insertion in mutant N5-G6 was intergenic between the putative *groES* and putative *groEL* genes (Figure 3.16). The location of the insertion meant that the transposon was able to disrupt one or both of these genes. On agar, the mutant showed increased sensitivity to both triclosan and *ortho*-phthalaldehyde and was resistant to chlorhexidine diacetate (Table 3.12). Increased resistance to the latter biocide was also confirmed in broth (Table 3.15). Results of the suspension tests and potassium leakage experiments showed that the mutant was more sensitive to the killing effect of triclosan and *ortho*-phthalaldehyde, leaking larger amounts of potassium than the wild type when subjected to the same biocides (Tables 3.16 and 3.17). Results also demonstrated that

chlorhexidine diacetate was less affective at killing the mutant than it was at killing the wild type (Table 3.16). The mutant leaked smaller amounts of potassium than did the wild type when subjected to chlorhexidine diacetate (Table 3.17). From these results it can be concluded that mutant N5-G6 had increased sensitivity to both the killing and the growth inhibitory effects of triclosan and *ortho*-phthalaldehyde, and had increased resistance to the same effects caused by exposure to chlorhexidine diacetate. Antibiotic susceptibility profiles (Table 3.18) showed that N5-G6 had increased resistance to four antibiotics, the two carbapenems, meropenem and imipenem, chloramphenicol and piperacillin. The mutant was also more sensitive than the wild type to the two aminoglycosides, amikacin and tobramycin, as well as (1/9) trimethoprim/sulfamethoxazole. A summary of information about the gene disrupted by transposon insertion in mutant N5-G6, and its surroundings is shown in Table 3.29.

Table 3.19. Summary of the mini-Tn*5* insertions identified on the *S. marcescens* Db11 mutants.

Mutant	Phenotype	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
8-C7	CHX^r, TRI^r	*carB*	62.4	Carbamoyl-phosphate synthase large subunit	PRK00320	97	0
10-E7, 3-F2 18-A3, 9-D5 3-A4 11-B8 7-C10 N2-B3 N2-F3	CPC^s, OPA^s CPC^s, CHX^s CPC^s, CHX^r, TRI^s OPA^s OPA^s, TRI^s CPC^s, CHX^s, OPA^s, TRI^s CHX^r, OPA^s, TRI^s	-	64.2	DeoR family transcriptional regulator	Pfam00455	76	7e-107
22-D5	CPC^s, OPA^s	*sdhD*	55.1	Succinate dehydrogenase hydrophobic membrane anchor	CD03494	86	5e-42
N4-F6, N2-A8, N5-G1	CPC^s, CHX^s	*wbdA*	47.3	MannosyltransferaseA/ glycosyltransferase group 1	Pfam00534	65	0
N2-F1	CHX^s						
N5-D9	TRI^s	*wbpZ*	44.5	Glycosyltransferase (WbpZ)	Pfam00534	58	1e-128
12-F6	CHX^s, OPA^s, TRI^s	*ompA*	54.3	Outer membrane protein A	PRK10808	99	0

Table 3.19. Summary of the mini-Tn*5* insertions identified on the *S. marcescens* Db11 mutants (continued).

Mutant	Phenotype	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
N5-B6	CPC^s, TRI^s	*nrdA*	57.9	Ribonucleotide-diphosphate reductase α subunit	CD01679	93	0
N3-B8	CPC^r, CHX^r, TRI^s	*ndpA*	60.9	Nucleoid-associated protein NdpA	PRK00378	87	3e-162
N5-B5	CPC^r, OPA^s, TRI^s	-	48.0	Pili assembly chaperone	COG3121	63	6e-75
N6-B2	CPC^s, OPA^s, TRI^s	*pfkA*	57.9	6-Phosphofructokinase	CD00763	97	3e-177
19-D3 10-B6	CPC^r, CHX^r, OPA^r CPC^s, OPA^r	*wzzE*	60.2	Lipopolysaccharide biosynthesis protein	PRK11638	92	4e-176
N5-G6	CHX^r, OPA^s, TRI^s	*groES* *groEL*	50.3 57.3	Co-chaperonin GroES Chaperonin GroEL	PRK05005 CD03344	87 99	4e-46 0
N1-C5	Same as the wild type	-	50.8	Hypothetical protein EcolE1_01003164	-	62	8e-110

TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate, G+C; guanine+cytosine, % identity; % amino acid identity, r; resistant, s; susceptible.

Table 3.20. Mapping of DNA around the transposon disrupted gene in mutants 10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3, and N2-F3.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3, N2-F3	-	58.8	Hypothetical protein Spro_0996	-	67	9e-79
	-	63.4	Integral membrane protein	COG0428	61	2e-46
	-	64.2	DeoR family transcriptional regulator	Pfam00455	76	7e-107
	-	64.6	Glyoxalase/bleomycin resistance protein	Pfam00903	81	2e-53
	-	66.5	LysR family transcriptional regulator	Pfam03466	92	1e-155
	-	61.1	Pirin domain protein	Pfam02678	94	4e-156
	-	59.7	Isochorismatase hydrolase	CD01012	96	4e-116

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.21. Mapping of DNA around the transposon disrupted gene in mutant 22-D5.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
22-D5	*gltA*	58.3	Citrate synthase	PRK05614	99	0
	sdhD	55.1	Succinate dehydrogenase hydrophobic membrane anchor protein	CD03494	86	5e-42
	sdhA	61.2	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit	PRK08958	97	0
	sdhB	56.3	Succinate dehydrogenase and fumarate reductase iron-sulfur protein	PRK05950	95	5e-134
	sucA	60.4	2-Oxoglutarate dehydrogenase E1 component	COG0567	92	0

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.22. Mapping of DNA around the transposon disrupted genes in mutants N4-F6, N2-A8, N5-G1, N2-F1, and N5-D9.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	wzt	44.2	ATP binding component of ABC-transporter	CD03220	78	0
	wbbD	45.5	Methyltransferase type 11	Pfam08241	54	7e-123
N4-F6, N2-A8, N5-G1, N2-F1	*wbdA*	47.3	Mannosyltransferase A/ glycosyltransferase group 1	Pfam00534	65	0
	wbdB	42.2	Mannosyltransferase B/glycosyltransferase group 1	Pfam00534	59	9e-134
N5-D9	*wbpZ*	44.5	Glycosyltransferase (WbpZ)	Pfam00534	58	1e-128
	gnd	57.4	6-Phosphogluconate dehydrogenase	PRK09287	93	0

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.23. Mapping of DNA around the transposon disrupted gene in mutant 12-F6.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	lonB	58.2	Lon protease	COG1067	88	0
	-	48.5	Hypothetical protein y2737	PRK05097	84	8e-57
12-F6	*ompA*	54.3	Outer membrane protein A	PRK10808	99	0
	sulA	49.9	Cell division inhibitor SulA	COG5404	97	1e-80
	-	58.9	Regulator of competence-specific genes	COG3070	75	1e-86
	-	59.1	Hypothetical membrane protein	Pfam05976	90	0

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.24. Mapping of DNA around the transposon disrupted gene in mutant N5-B6.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	gyrA	60.0	DNA gyrase subunit A	PRK05560	99	0
	ubiG	59.6	3-Demethylubiquinone-9 3-methyltransferase	Pfam08241	85	8e-122
N5-B6	*nrdA*	57.9	Ribonucleotide-diphosphate reductase alpha subunit	PRK05134	93	0
	nrdB	56.4	Ribonucleotide-diphosphate reductase beta subunit	PRK09101	94	0

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.25. Mapping of DNA around the transposon disrupted gene in mutant N3-B8.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	bcr	61.7	Bicyclomycin resistance protein/multidrug efflux system	PRK11102	71	2e-141
	rsuA	61.4	Ribosomal small subunit pseudouridine synthase A	CD02553	88	5e-117
	-	64.2	DNA or RNA helicases of superfamily II	COG1061	85	0
	rplY	47.1	50S ribosomal protein L25	PRK05943	78	6e-42
N3-B8	*ndpA*	60.9	Nucleoid-associated protein NdpA	PRK00378	87	3e-162
	yejM	60.4	Sulphatase	COG3083	64	0
	-	53.0	Hypothetical protein HS_1396	Pfam03681	56	2e-38
	-	37.7	Acyltransferase transmembrane protein	COG1835	30	2e-24

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.26. Mapping of DNA around the transposon disrupted gene in mutants N5-B5 and N1-C5.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	yccA	57.7	Carrier/transport protein	PRK10447	79	5e-73
	safA	51.8	Mannose sensitive type I fimbriae major structural subunit	COG3539	94	3e-90
N5-B5	-	48.0	Pili assembly chaperone	COG3121	63	6e-75
	-	55.8	Fimbrial biogenesis outer membrane usher protein	Pfam00577	64	0
N1-C5	-	50.8	Hypothetical protein EcolE1_01003164	-	62	8e-110
	-	43.3	DNA-damage-inducible protein J	COG3077	38	6e-06
	-	48.6	Two component transcriptional regulator, LuxR family	Pfam00196	27	7e-06
	soxR	60.0	Redox-sensitive transcriptional activator soxR	CD01110	63	8e-50
	mexH	64.6	RND efflux membrane fusion protein precursor	PRK11556	63	4e-96

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.27. Mapping of DNA around the transposon disrupted gene in mutant N6-B2.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	tipA	59.7	Triosephosphate isomerase proteins	CD00311	93	4e-119
	sbp	57.2	Periplasmic sulfate-binding protein	COG1613	93	4e-172
N6-B2	*pfkA*	57.9	6-Phosphofructokinase	CD00763	97	3e-177
	fieF	59.7	Ferrous iron efflux protein F	PRK09509	82	1e-132
	cpxP	55.2	Periplasmic repressor of the Cpx envelope stress response pathway	PRK10363	92	4e-46
	cpxR	59.0	CpxR transcriptional regulator	PRK10955	91	1e-104

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.28. Mapping of DNA around the transposon disrupted gene in mutants 19-D3 and 10-B6.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	rho	54.6	Rho transcription termination factor	PRK09376	99	0
	rfe	55.1	UDP-alpha-*N*-acetylglucosaminyl 1-phosphatetransferase	COG0472	95	9e-168
19-D3, 10-B6	*wzzE*	60.2	Lipopolysaccharide biosynthesis protein	PRK11638	92	4e-176
	wecB/rffE	59.4	UDP-*N*-acetylglucosamine 2-epimerase	COG0381	86	0
	wecC/rffD	59.7	UDP-*N*-acetyl-D-mannosamine dehydrogenase	PRK11064	94	0

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.29. Mapping of DNA around the transposon disrupted gene in the intergenic mutant N5-G6.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	aspA	57.1	Aspartate ammonia-lyase	CD01357	98	0
	fxsA	60.0	Suppressor of F plasmid exclusion of phage T7	COG3030	87	3e-67
N5-G6	*groES*	50.3	Co-chaperonin GroES	PRK05005	87	4e-46
	groEL	57.3	Chaperonin GroEL	CD03344	99	0
	cheD	62.7	Methyl-accepting chemotaxis protein	PRK09793	42	1e-85

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

Table 3.30. Mapping of DNA around the transposon disrupted gene in mutant 8-C7.

Mutant	Disrupted gene	% G+C	Protein putative function	Protein ID	% Identity	E-value
	dapB	61.5	Dihydrodipicolinate reductase	COG0289	89	2e-125
	carA	59.1	Carbamoyl-phosphate synthase small subunit	COG0505	95	0
8-C7	*carB*	62.4	Carbamoyl-phosphate synthase large subunit	PRK00320	97	0
	yabF	58.0	NAD(P)H oxidoreductase	PRK00871	67	3e-64

Site of the mini-Tn*5* insertion is highlighted in red. G+C; guanine+cytosine, % identity; % amino acid identity.

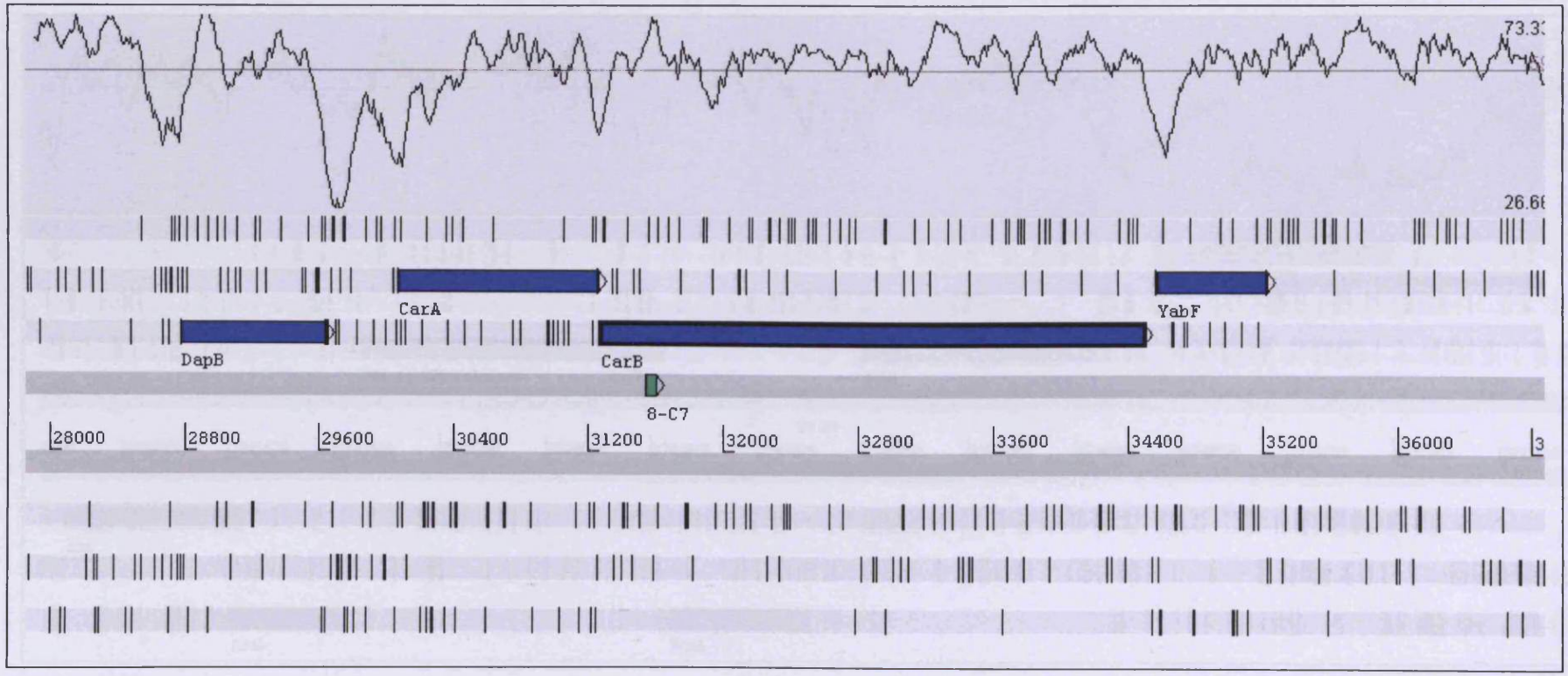


Figure 3.12. Mapping of DNA around the transposon disrupted gene in mutant 8-C7

Artemis (929) screen view of the putative carbamoyl-phosphate synthase large subunit mutant 8-C7 and the surrounding genes marked in blue. Full details of the mutants and the genes are given in Table 3.30. Position of the mini-Tn5 insertion into the *S. marcescens* Db11 genome is indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. DapB; dihydrodipicolinate reductase, CarA; carbamoyl-phosphate synthase small subunit, CarB; carbamoyl-phosphate synthase large subunit, YabF; NAD(P)H oxidoreductase.

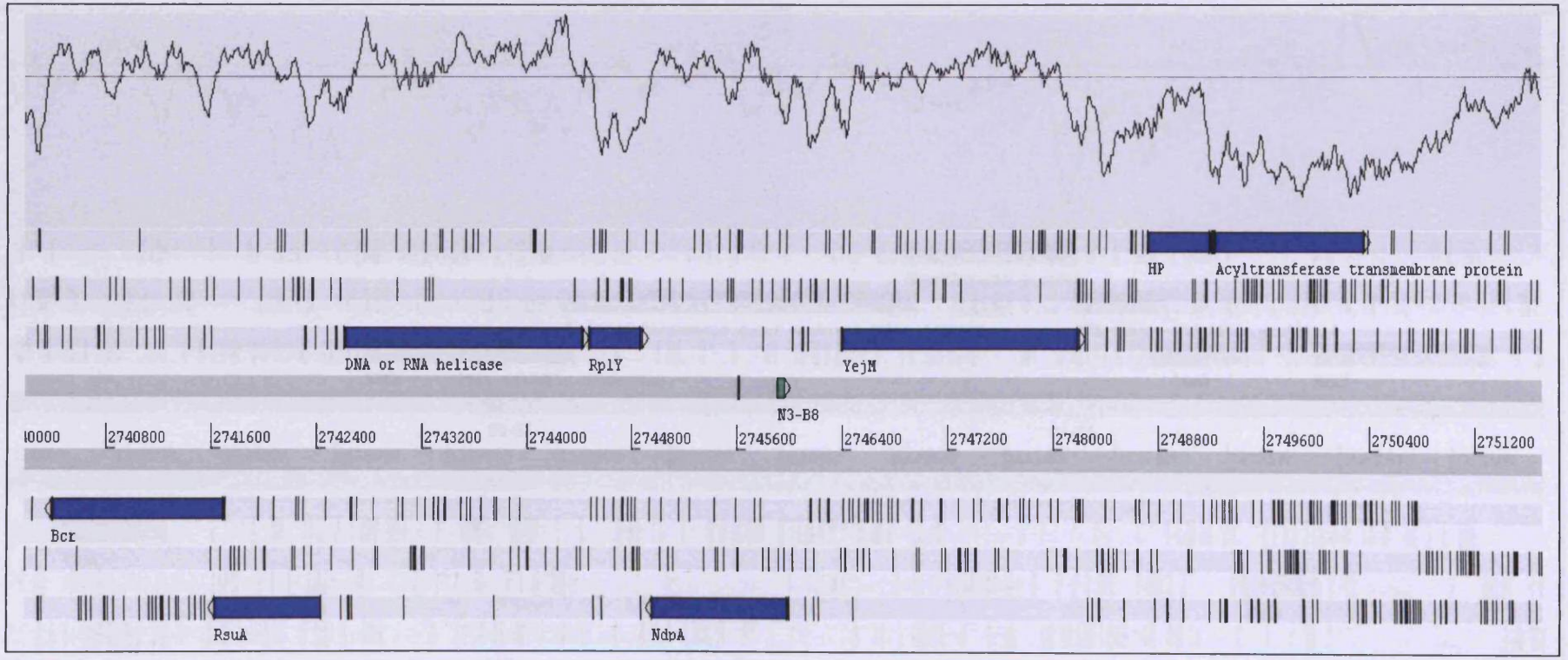


Figure 3.13. Mapping of DNA around the transposon disrupted gene in mutant N3-B8

Artemis (929) screen view of the putative nucleoid-associated protein (NdpA) mutant N3-B8 and the surrounding genes marked in blue. Full details of the mutant and the genes are given in Table 3.25. Position of the mini-Tn5 insertion into the *S. marcescens* Db11 genome is indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. Bcr; bicyclomycin resistance protein, RsuA; ribosomal small subunit pseudouridine synthase A, RplY; 50S ribosomal protein L25, NdpA; nucleoid-associated protein, YejM; sulphatase, HP; hypothetical protein HS_1396.

Figure 3.14. Mapping of DNA around the transposon disrupted genes in mutants N5-B5 and N1-C5

Artemis (929) screen view of the putative pili assembly chaperone mutant N5-B5 and the putative hypothetical protein EcolE1_01003164 control mutant N1-C5, and the surrounding genes marked in blue. Full details of the mutants and the genes are given in Table 3.26. Positions of the mini-Tn5 insertions into the *S. marcescens* Db11 genome are indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. YccA; carrier/transport protein, SafA; mannose sensitive type I fimbriae major structural subunit, LuxR; two component transcriptional regulator (LuxR family), SoxR; redox-sensitive transcriptional activator, MexH; RND efflux membrane fusion protein precursor.

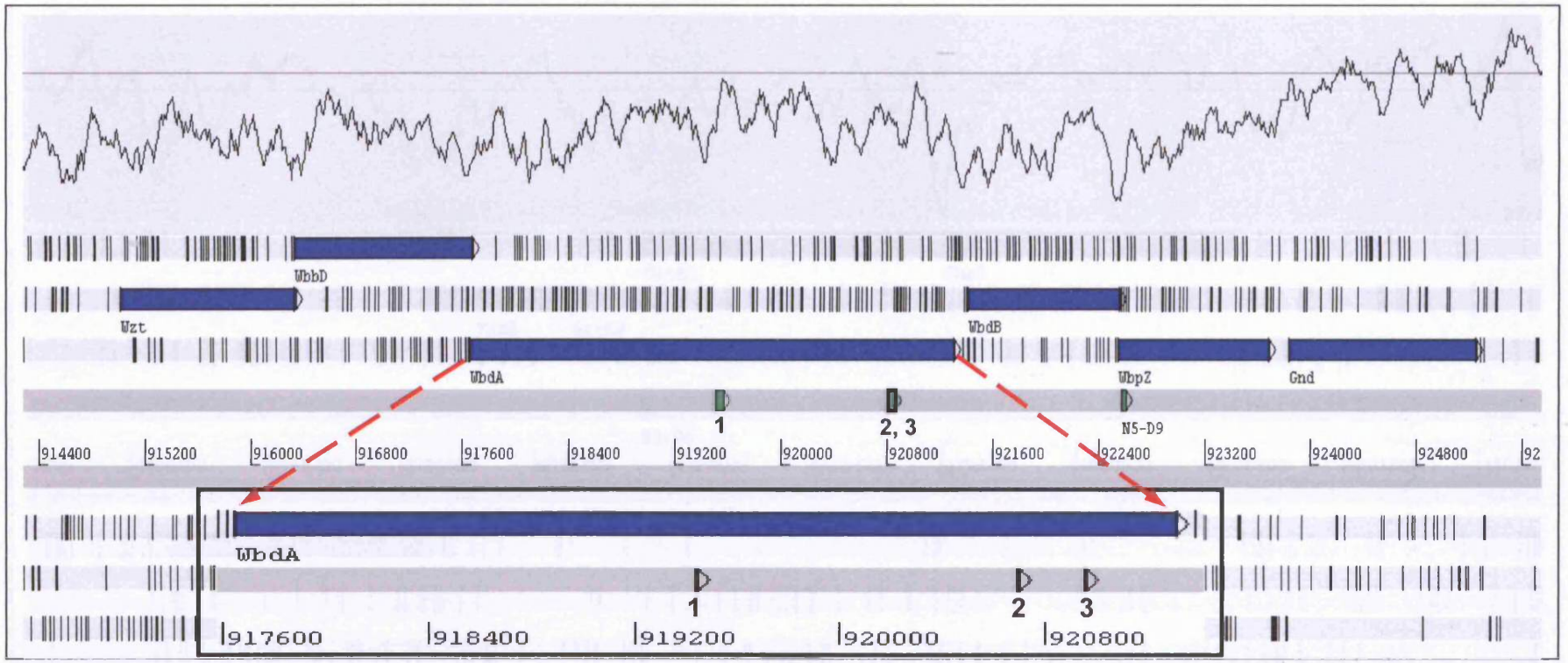


Figure 3.15. Mapping of DNA around the transposon disrupted genes in mutants N4-F6, N5-G1, N2-A8, N2-F1, and N5-D9

Artemis (929) screen view of the putative mannosyltransferase A (WbdA) mutants N4-F6, N5-G1, N2-A8, N2-F1 and the putative glycosyltransferase (WbpZ) mutant N5-D9 and the surrounding genes marked in blue. Full details of the mutants and the genes are given in Table 3.22. Positions of the mini-Tn5 insertions into the *S. marcescens* Db11 genome are indicated in green. Site 1; transposon insertion in mutant N4-F6, site 2; transposon insertion in mutants N5-G1 and N2-A8, site 3; transposon insertion in mutant N2-F1. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. The ORFs *wzt*, *wbbD*, *wbdA*, *wbdB* and *wbpZ* were all part of a low G+C content region of the *S. marcescens* Db11 that extended over 14,000 bp. Wzt; ATP binding component of ABC-transporter, WbbD; methyltransferase type 11, WbdA; mannosyltransferase A, WbdB; mannosyltransferase B, WbpZ; glycosyltransferase, Gnd; 6-phosphogluconate dehydrogenase.

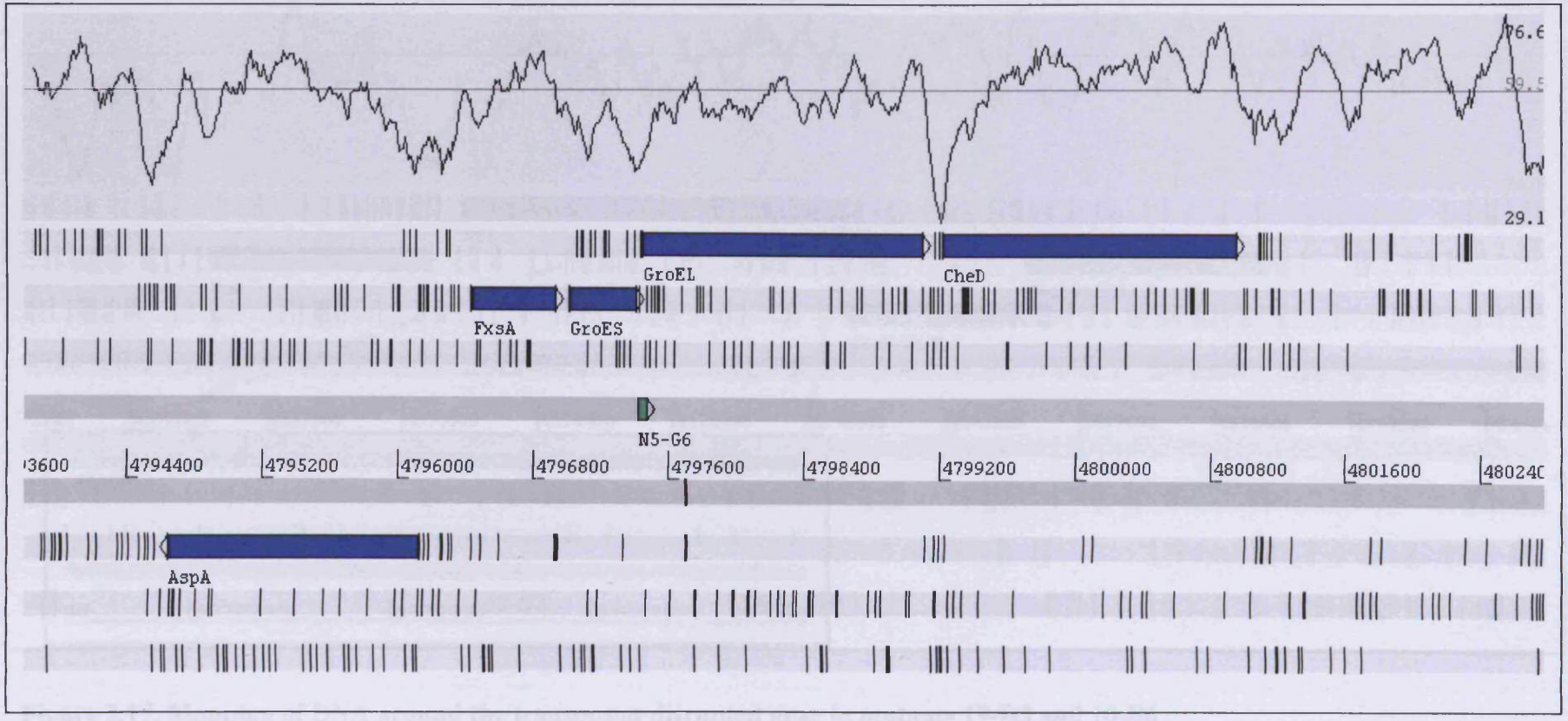


Figure 3.16. Mapping of DNA around the transposon disrupted gene in mutant N5-G6

Artemis (929) screen view of the putative chaperonin mutant N5-G6 and the surrounding genes marked in blue. Full details of the mutant and the genes are given in Table 3.29. Position of the mini-Tn5 insertion into the *S. marcescens* Db11 genome is indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. AspA; aspartate ammonia-lyase, FxsA; suppressor of F plasmid exclusion of phage T7, GroES; co-chaperonin, GroEL; chaperonin, CheD; methyl-accepting chemotaxis protein.

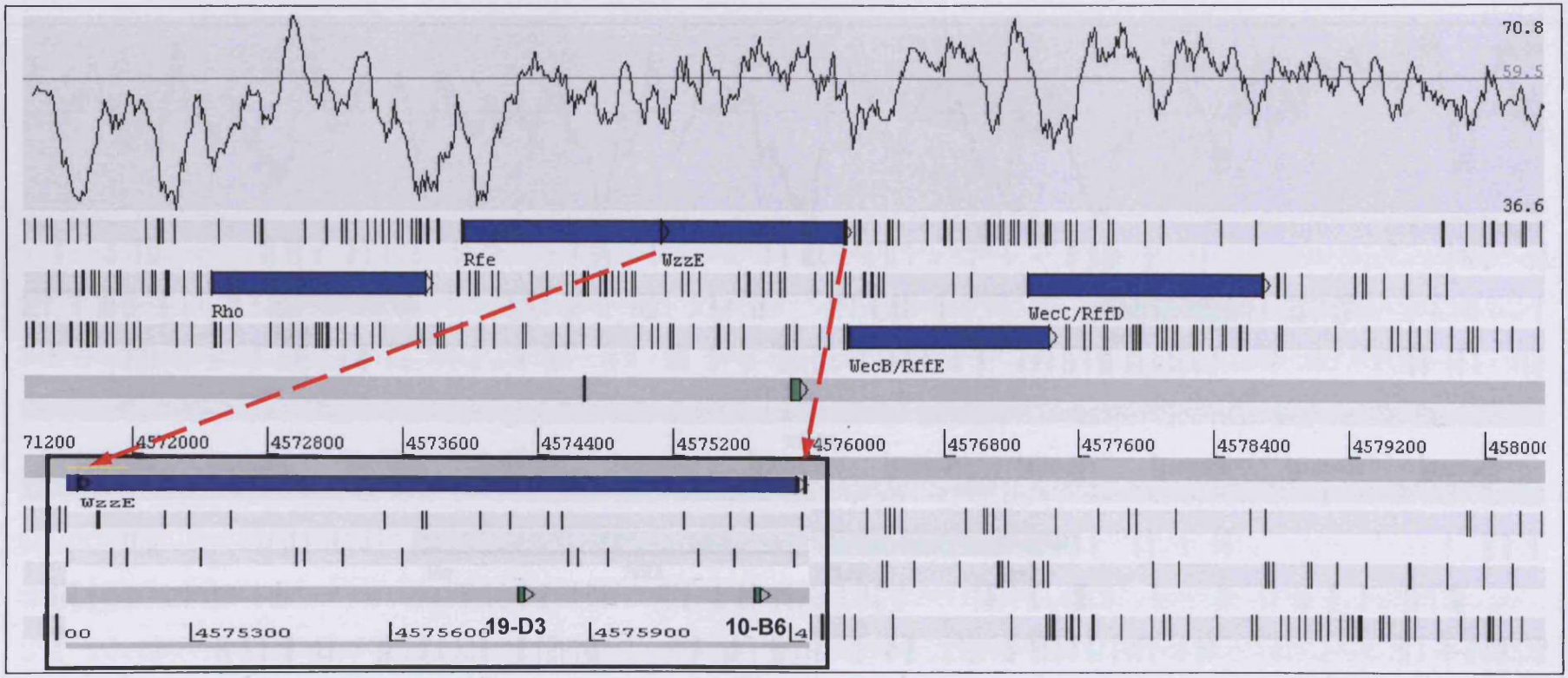


Figure 3.17. Mapping of DNA around the transposon disrupted gene in mutants 19-D3 and 10-B6

Artemis (929) screen view of the putative lipopolysaccharide biosynthesis protein (WzzE) mutants 19-D3 and 10-B6, and the surrounding genes marked in blue. Full details of the mutants and the genes are given in Table 3.28. Positions of the mini-Tn5 insertions into the *S. marcescens* Db11 genome are indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. Rho; transcription termination factor, Rfe; UDP-alpha-*N*-acetylglucosaminyl 1-phosphatetransferase, WzzE; lipopolysaccharide biosynthesis protein, WecB; UDP-*N*-acetylglucosamine 2-epimerase, WecC; UDP-*N*-acetyl-D-mannosamine dehydrogenase.

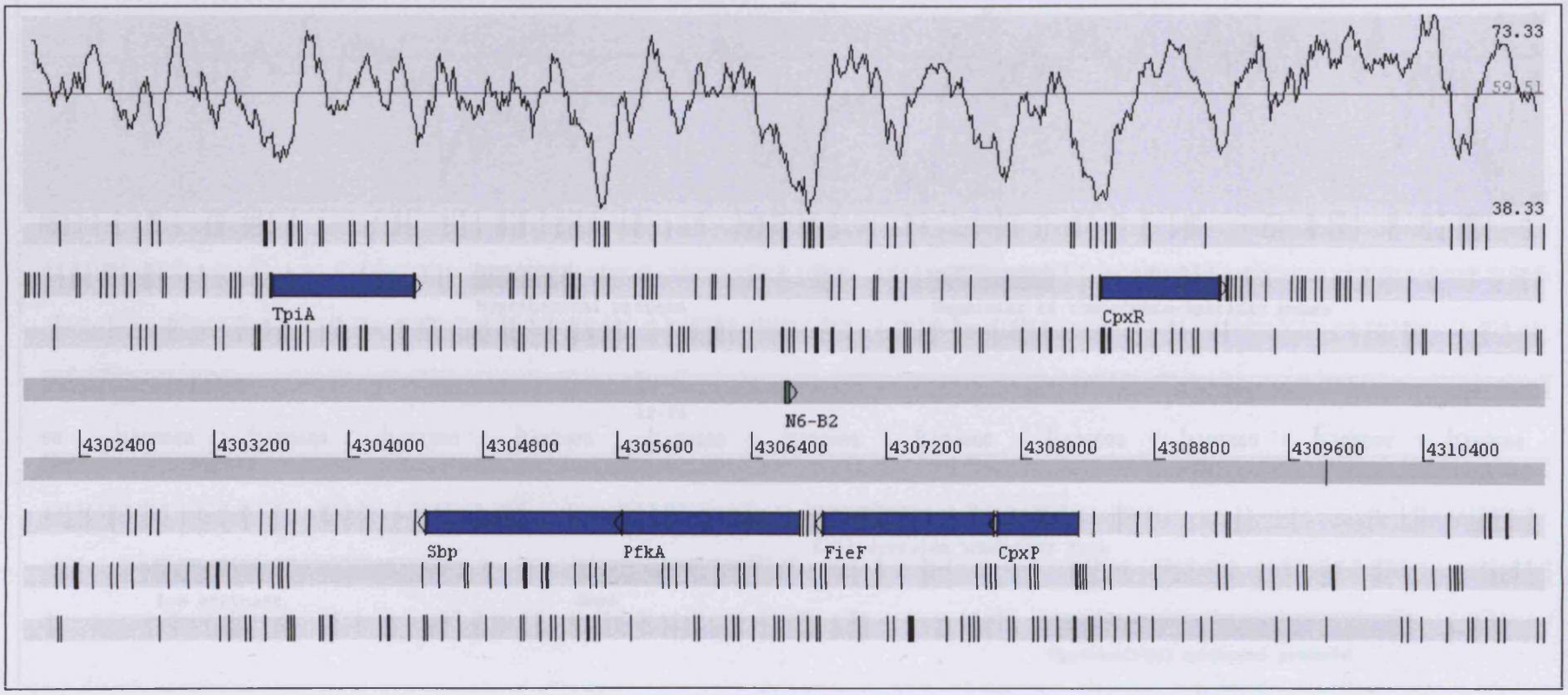


Figure 3.18. Mapping of DNA around the transposon disrupted gene in mutant N6-B2

Artemis (929) screen view of the putative 6-phosphofructokinase (PfkA) mutant N6-B2, and the surrounding genes marked in blue. Full details of the mutant and the genes are given in Table 3.27. Position of the mini-Tn5 insertion into the *S. marcescens* Db11 genome is indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. TpiA; triosephosphate isomerase proteins, Sbp; periplasmic sulfate-binding protein, PfkA; 6-phosphofructokinase, FieF; ferrous iron efflux protein F, CpxP; periplasmic repressor of the Cpx envelope stress response pathway, CpxR; transcriptional regulator.

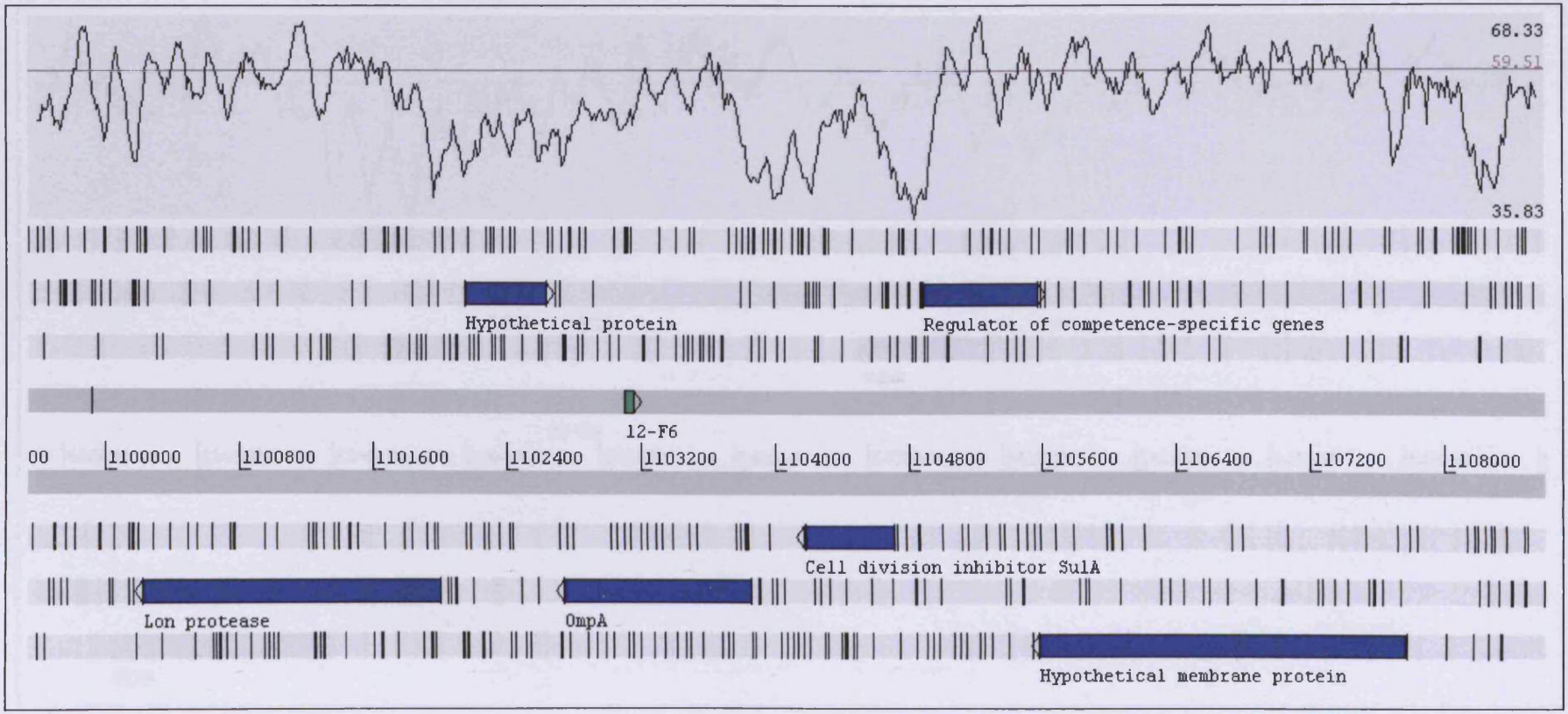


Figure 3.19. Mapping of DNA around the transposon disrupted gene in mutant 12-F6

Artemis (929) screen view of the putative outer membrane protein A (OmpA) mutant 12-F6 and the surrounding genes marked in blue. Full details of the mutant and the genes are given in Table 3.23. Position of the mini-Tn5 insertion into the *S. marcescens* Db11 genome is indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF.

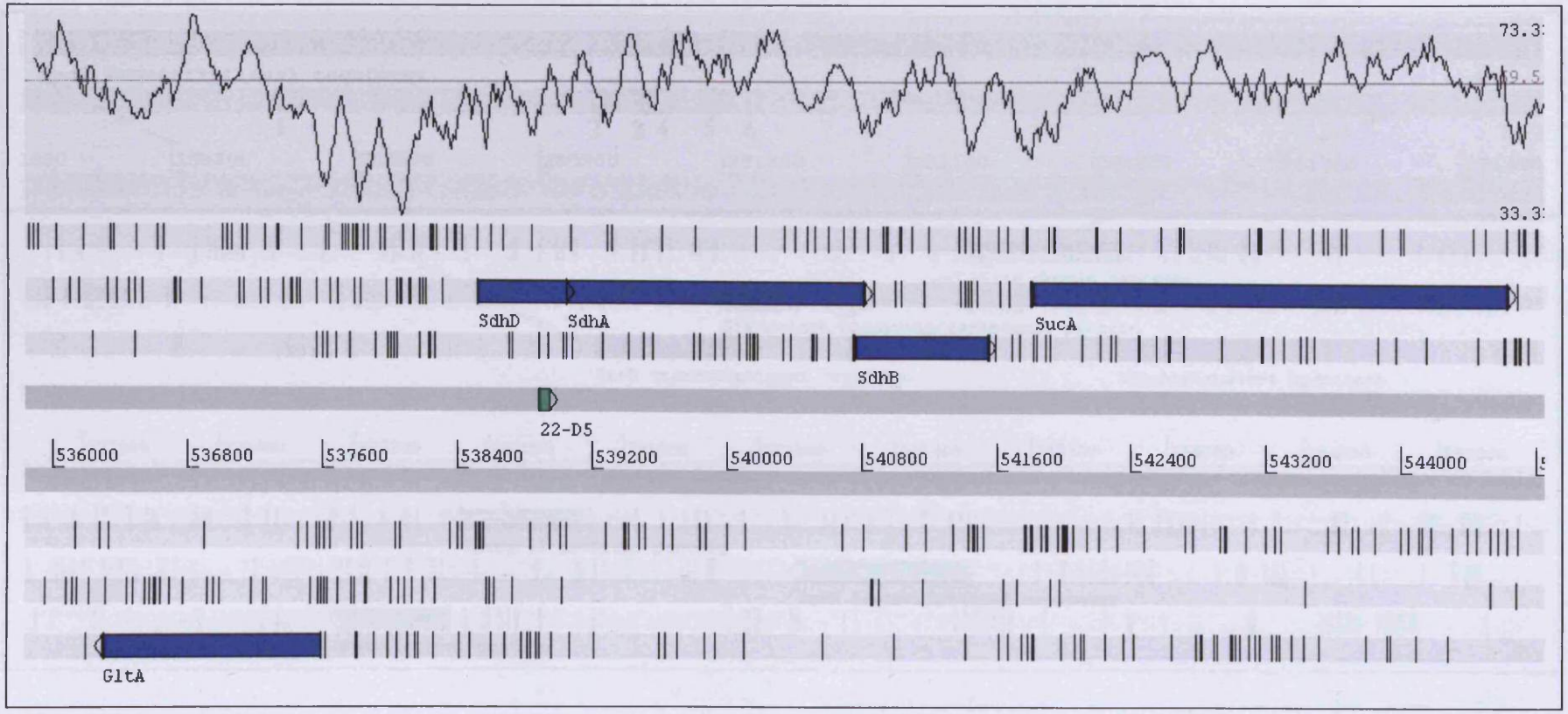


Figure 3.20. Mapping of DNA around the transposon disrupted gene in mutant 22-D5

Artemis (929) screen view of the putative succinate dehydrogenase hydrophobic membrane anchor protein (SdhD) mutant 22-D5 and the surrounding genes marked in blue. Full details of the mutant and the genes are given in Table 3.21. Position of the mini-Tn5 insertion into the *S. marcescens* Db11 genome is indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. GltA; citrate synthase, SdhD; succinate dehydrogenase hydrophobic membrane anchor protein, SdhA; succinate dehydrogenase/fumarate reductase, flavoprotein subunit, SdhB; succinate dehydrogenase and fumarate reductase iron-sulfur protein, SucA; 2-oxoglutarate dehydrogenase E1 component.

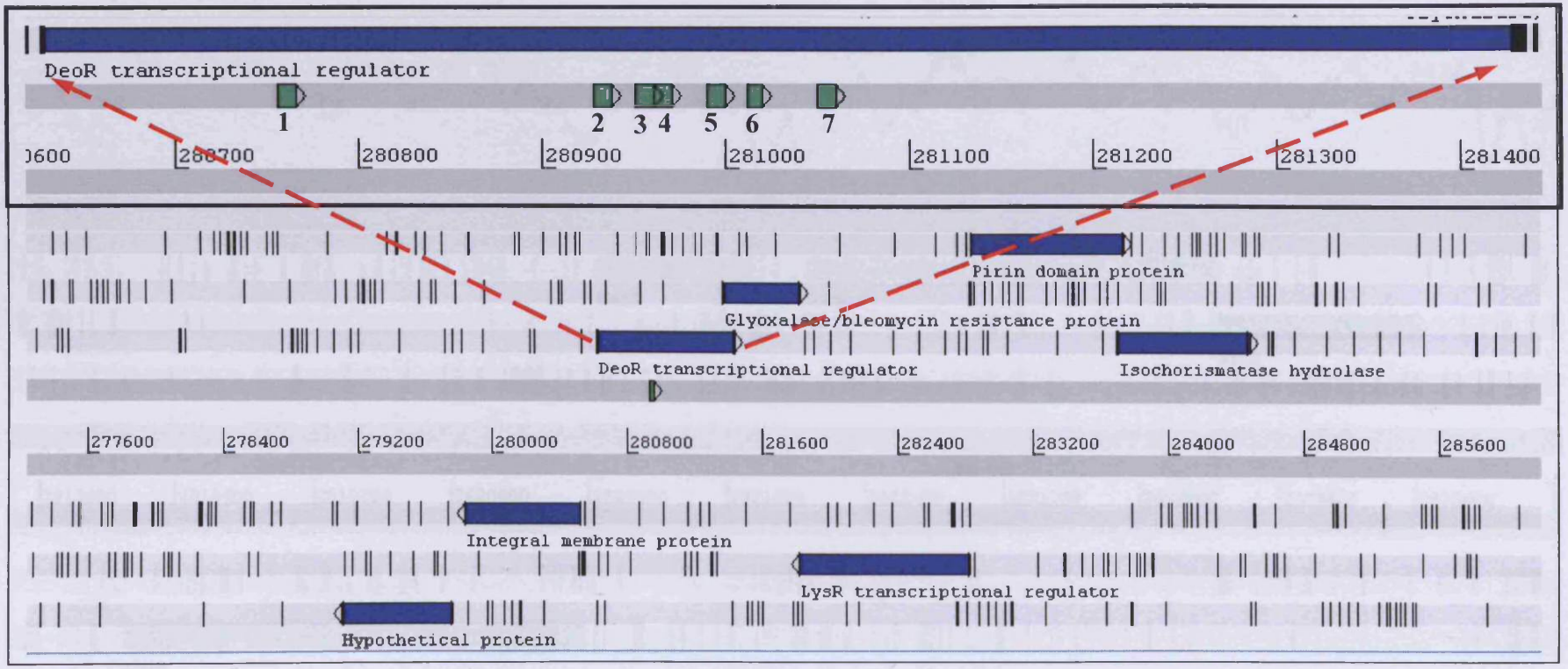


Figure 3.21. Mapping of DNA around the transposon disrupted gene in mutants 10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3 and N2-F3

Artemis (929) screen view of the putative DeoR family transcriptional regulator mutants 10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3 and N2-F3 and the surrounding genes marked in blue. Full details of the mutants and the genes are given in Table 3.20. The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. Positions of the mini-Tn5 insertions into the *S. marcescens* Db11 genome are indicated in green. Site 1; transposon insertion in mutant 3-A4, site 2; transposon insertion in mutant N2-B3, site 3; transposon insertion in mutant N2-F3, site 4; transposon insertion in mutant 7-C10, site 5; transposon insertion in mutants 10-E7 and 3-F2, site 6; transposon insertion in mutant 11-B8, site 7; transposon insertion in mutants 18-A3 and 9-D5.

Figure 3.22. Mapping of DNA around the transposon disrupted gene in mutant N5-B6

Artemis (929) screen view of the putative ribonucleotide-diphosphate reductase alpha subunit (NrdA) mutant N5-B6 and the surrounding genes marked in blue. Full details of the mutant and the genes are given in Table 3.24. Position of the mini-Tn5 insertion into the *S. marcescens* Db11 genome is indicated in green. The DNA G+C content plot is shown on top of the screen view, and the line crossing the graph represents the mean G+C content of the *S. marcescens* Db11 genome (59.5%). The blue rectangles represent open reading frames (ORFs) derived from the automatic annotation of the Db11 genome, and the vertical lines indicate stop codons. The putative protein products are shown under the corresponding ORF. GyrA; DNA gyrase subunit A, UbiG; 3-demethylubiquinone-9 3-methyltransferase, NrdA; ribonucleotide-diphosphate reductase alpha subunit, NrdB; ribonucleotide-diphosphate reductase beta subunit.

3.4.14 Detailed analysis of the *ompA* biocide mutant

Mutant 12-F6 showed increased sensitivity to triclosan, *ortho*-phthalaldehyde, and chlorhexidine diacetate in the initial biocide screenings. The disrupted gene in the mutant was identified as the putative *ompA* gene encoding for the outer membrane protein A in *S. marcescens* Db11 (Table 3.23, Figure 3.19). The mutant phenotype was investigated further by determining its biocides agar and broth MICs, biocides lethality effects on the mutant, potassium leakage tests, and the mutant's antibiotic susceptibility profile. On agar, the mutant showed 10%, 17% and 18% increase in susceptibility to *ortho*-phthalaldehyde, chlorhexidine diacetate and triclosan respectively (Table 3.31). Statistical analysis showed that the differences in MIC values between the wild type *S. marcescens* Db11 and the *ompA* mutant were only significant at 60% confidence level for chlorhexidine diacetate and *ortho*-phthalaldehyde, and at 80% confidence level for triclosan.

However, it was noted that the decrease in the agar MIC values for these biocides was reproducible and generally constant in all our replica tests. The results of the agar MIC ratios (Table 3.13) confirmed the above observation. They showed that for chlorhexidine diacetate the ratio values between the mutant and the wild type for the maximum and the minimum MICs generated in our replica tests were 0.8 and 0.87 respectively. Similarly, the ratio values for the same above parameters were 0.85 and 0.78 for triclosan and 0.87 and 0.92 for *ortho*-phthalaldehyde. These results showed that although the increase in susceptibility of the *ompA* mutant to *ortho*-phthalaldehyde, triclosan and chlorhexidine diacetate, was small, it was nevertheless reproducible and genuine. Broth MICs for the *ompA* mutant were also determined for chlorhexidine diacetate and cetylpyridinium chloride (Table 3.31). For the latter, there was no significant difference between the MIC values for the *ompA* mutant and the wild type *S. marcescens* Db11. For chlorhexidine diacetate on the other hand, the mutant showed a 20% increase in susceptibility to this biocide compared to the wild type, and this increase in sensitivity was statistically significant at the 95% confidence level.

The lethality effect of the four biocides on the *ompA* mutant was investigated by suspension (section 3.3.5.3) and potassium leakage tests (section 3.3.6). Results of the

former (Table 3.32) showed that the number of the *ompA* mutant cells killed after exposure to 8 μg/ml cetylpyridinium chloride for 10 min was not statistically significantly different from that of the wild type. On the other hand, exposure to 20 μg/ml chlorhexidine diacetate for the same period of time cause 7.3% more cell death in the mutant compared with the wild type. A 5 min exposure to 4000 μg/ml triclosan led to 44% more mutant cells killed than it did for the wild type. The biggest increase in cell mortality (247%) was observed after mutant exposure to 50 μg/ml *ortho*-phthalaldehyde for 5 min. All the above increases in cell mortality compared with the wild type were statistically significant at the 95% confidence level.

The other parameter used to determine the lethality effect of the above concentrations of biocide on the mutant was to measure the amount of potassium leaked from a biocide-treated suspension of mutant and compare it with the wild type. The results (Table 3.32) showed that all three biocides, chlorhexidine diacetate, triclosan and *ortho*-phthalaldehyde, at the above concentrations and exposure times, lead to more potassium to be released from the mutant suspensions than the wild type. These increases were of 20%, 38%, and 64% for triclosan, *ortho*-phthalaldehyde and chlorhexidine diacetate respectively. The increases in the extracellular potassium in the mutants suspensions compared with the wild type were all statistically significant at the 95% confidence level.

The antibiotic susceptibility profile of the *ompA* mutant was determined for ten antibiotics. The results (Table 3.33) showed that compared with the wild type, the *ompA* mutant was more resistant to amikacin, tobramycin and imipenem. It also showed increased sensitivity to azithromycin, piperacillin, and chloramphenicol. All these changes in MIC values were statistically significant at the 95% confidence level. No changes in MICs were observed for the mutant with ceftazidime, meropenem, ciprofloxacin or (1/19) trimethoprim/sulfamethoxazole. The mutant was also sensitive to 10 μg/ml gentamicin but resistant to 100 μg/ml kamamycin and to 100 units/ml polymyxin B.

3.4.14.1 Complementation of the *ompA* mutant

3.4.14.1.1 Complementation steps

To confirm that the phenotypic change in the *ompA* mutant was due to the disruption of the *ompA* gene, complementation of the mutant was attempted as described in section 3.3.13. A construct, SABE*ompA*, was generated by ligation of a *S. marcescens* Db11 DNA fragment containing the *ompA* gene, its putative promoter region, and its translation start and stop signals as described by Braun and Cole (95) (Figure 3.23), to the pURF047 cloning vector. Tri-parental mating (section 3.3.13.6) was used to introduce the construct into the *ompA* mutant and into the wild type *S. marcescens* Db11 to determine the effect of harbouring an extra copy of the *ompA* gene on the organism. Moreover, tri-parental mating was used to introduce a copy of the cloning vector pURF047 into both the wild type *S. marcescens* Db11 and the *ompA* mutant to determine whether the presence of the vector alone would influence the mutant or the wild type phenotype.

Three *ompA* mutant derivatives (MS1, MS2, and MS3) and one wild type derivative (WS1) were isolated and the successful introduction of the SABE*ompA* into these transconjugants was checked using PCR. The results (Figure 3.24) confirmed that the *ompA* gene construct had been successfully introduced into the MS2 and MS3 transconjugants. Further investigations to determine whether the phenotypic complementation of the *ompA* mutant was successful (section 3.3.13.7) were then undertaken.

3.4.14.1.2 Phenotypic complementation analysis

The results from the *ompA* PCR amplification suggested that in transconjugants MS2 and MS3, *ompA* complementation had been successful in terms of introducing a wild-type copy of the gene into the mutant. To investigate further, phenotypic complementation analysis was performed on both transconjugants to check for restoration of the wild type phenotype. No noticeable difference in growth was seen in the wild type or in the *ompA* mutant cells containing the pURF047 vector or in the transconjugants compared to the wild type *S. marcescens* Db11. This suggested that

the presence of the pURF047 vector or the introduced copy of the *ompA* gene in the cells did not affect their growth fitness. The results of agar biocide MICs on the MS2 and MS3 transconjugants (Table 3.34) showed no significant difference in their MIC values from those of the *ompA* mutant. This meant that there was no restoration of the wild type phenotype in these transconjugants. Moreover the results also showed that the presence of the pURF047 vector or the introduced extra copy of the *ompA* in the wild type had not affected biocide susceptibility.

3.4.14.2 Reverse transcriptase reaction (RT-PCR)

The complementation analysis showed that there was no restoration of the wild type phenotype in the *ompA*-containing transconjugants MS2 and MS3. To investigate this, a study of the *ompA* expression at the mRNA level was undertaken. A reverse transcriptase (RT) PCR was performed on cDNA generated from RNA extracted from both the wild type *S. marcescens* Db11 and the *ompA* mutant and all their derivatives (section 3.3.13.8). The result of the RT-PCR (Figure 3.25) showed a PCR product of the expected size was generated with the wild type *S. marcescens* Db11 and its derivative SW1, but also in transconjugant MS3. No PCR product was seen with the *ompA* mutant or its complemented strains MS1 and MS2. MS3 was the only complemented mutant to express the *ompA* transcript.

Evidence of the *ompA* transcription in the wild type and its derivatives (*S. marcescens* Db11::pURF047 and WS1) was not surprising as they all contained the original functional *ompA* gene. Similarly, as the *ompA* mutant and the *ompA* mutant::pURF047 derivative both contain a disrupted copy of the *ompA* gene, lack of evidence of transcription of the gene was also not surprising. On the other hand, in the MS3 transconjugant which contained an intact *ompA* copy as well as the disrupted one, there was evidence that the *ompA* gene was being transcribed but still demonstrated no restoration of the wild type phenotype. This indicated that no protein synthesis from this RNA occurred.

3.4.14.3 Whole cell protein electrophoresis

To investigate further, whole cell protein electrophoresis was performed on the wild type *S. marcescens* Db11, the *ompA* mutant and all their derivatives (section 3.3.9) to check whether the OmpA protein was being translated in these cells and could be observed at the protein level. Bioinformatics analysis showed that the *S. marcescens* Db11 OmpA has a molecular weight of ≈ 39-kDa similar to that already reported by Braun and Cole (95). The results of the protein electrophoresis (Figure 3.26) showed that as expected the OmpA protein was present in the wild type *S. marcescens* Db11 and all its derivatives. The OmpA however was not present in the *ompA* mutant or in any of its derivatives including MS3. The size of the protein absent in the *ompA* mutant and its derivatives was ≈ 30-kDa, smaller than the OmpA predicted size. This could be due to protein processing in the cell or to the gel conditions. Results of the protein electrophoresis suggested that although the *ompA* gene was being transcribed in MS3, the OmpA protein was not being translated, which explained the MS3 phenotype. There was no evidence of increased expression of the OmpA in the SW1 transconjugant even though it contained and extra copy of the *ompA* gene.

3.4.14.4 Biocide exposure and OmpA expression in *S. marcescens* Db11

Increased sensitivity of the *ompA* mutant to triclosan, *ortho*-phthalaldehyde, and chlorhexidine diacetate, suggested that the OmpA protein may be linked to resistance to these biocides. An attempt was made to determine whether exposure to increasing concentrations of these biocides induced or increased the OmpA expression in *S. marcescens* Db11. Cultures of the latter were grown in the presence of 1, 4, 8, and 14 μg/ml chlorhexidine diacetate and in 10, 20, 30, and 40 μg/ml triclosan. Proteins were extracted from cells and analyses (section 3.3.9), to produce protein profiles for each of the cultures. Visual analysis of the results (Figure 3.27) showed that there was no significant increasing in the OmpA expression in cells exposed to biocides. The levels of OmpA seemed to be similar to that of the control cultures, even at increased concentrations of the two biocides. There was also no change in expression of any other proteins.

Table 3.31. Agar and broth MICs for both wild type *S. marcescens* Db11 and the *ompA* mutant.

Biocide	Broth (TSB) MIC µg/ml (± SD)		Agar (TSA) MIC µg/ml (± SD)	
	***S. marcescens* Db11**	***ompA* mutant**	***S. marcescens* Db11**	***ompA* mutant**
CPC	5.5 (0.70)	5.5 (0.70)	100 (28.3)	100 (28.3)
CHX	7.5 (0.70)	6 (1.41)	18 (2.83)	15 (1.41)
TRI	Nd	Nd	3900 (141)	3200 (283)
OPA	Nd	Nd	3000 (283)	2700 (141)

SD; standard deviation, TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate, Nd; not determined.
Only values that were statistically significantly different at 95% confidence level from those of the wild type are highlighted (red).

Table 3.32. Comparison between *S. marcescens* Db11 and the *ompA* mutant using two biocide lethality measuring parameters.

Biocide	Suspension tests [Log_{10} reduction in cfu/ml]		Potassium leakage tests [Potassium released (ppm)]	
	***S. marcescens* Db11**	***ompA* mutant**	***S. marcescens* Db11**	***ompA* mutant**
CPC	3.13	2.88	0.10	0.10
CHX	2.57	2.76	1.07	1.76
TRI	3.11	4.47	0.80	0.96
OPA	1.18	4.09	0.50	0.69

TRI; triclosan at 4000 µg/ml after 5 min exposure, OPA; *ortho*-phthalaldehyde at 50 µg/ml after 5 min exposure, CPC; cetylpyridinium chloride at 8 µg/ml after 10 min exposure, CHX; chlorhexidine diacetate at 20 µg/ml after 10 min exposure. Only values that were statistically significantly different at 95% confidence level from those of the wild type are highlighted (red).

Table 3.33. Antibiotic MICs for the wild type *S. marcescens* Db11 and the *ompA* mutant.

Antibiotic	MIC (µg/ml)		Mutant sensitivity	
	S. marcescens Db11	*ompA* mutant	Compared to BSAC MIC breakpoints*	Compared to *S. marcescens* Db11
Aminoglycosides				
Amikacin	8	12	R	R
Tobramycin	25.1	56	R	R
Cephalosporins				
Ceftazidime	0.71	0.85	S	Nc
Carbapenems				
Imipenem	0.59	2.25	S	R
Meropenem	0.12	0.109	S	Nc
Macrolides				
Azithromycin	>256	1.01.3	-	S
Penicillins				
Piperacillin	2	1.5	S	S
Quinolones				
Ciprofloxacin	0.17	0.19	S	Nc
Sulfonamides***				
Trimethoprim/ sulfamethoxazole	0.65	0.46	-	Nc
Others				
Chloramphenicol	24	8	S**	S

Statistically significant increase in resistance (blue) or susceptibility (red) are highlighted
* Susceptibility based on BSAC MIC breakpoints for *Enterobacteriaceae* (640).
** The only change in sensitivity based on BSAC MIC breakpoints
*** Trimethoprim/sulfamethoxazole (1/19).
R; resistant, S; susceptible, Nc; no change in sensitivity

Table 3.34. Phenotypic complementation analysis of the *ompA* mutant.

Organism	Agar MICs µg/ml			
	CPC	CHX	TRI	OPA
S. marcescens Db11	100	18	3900	3000
S. marcescens Db11:: pURF047	100	18	3900	3000
ompA mutant	100	15	3200	2700
ompA mutant:: pURF047	100	15	3200	2700
SW1	100	18	3900	3000
MS2	100	15	3200	2700
MS3	100	15	3200	2700

TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate, SW1; *S. marcescens* Db11:: SABE*ompA*, MS2 and MS3; *ompA* mutant:: SABE*ompA*. Increased susceptibility is highlighted in red.

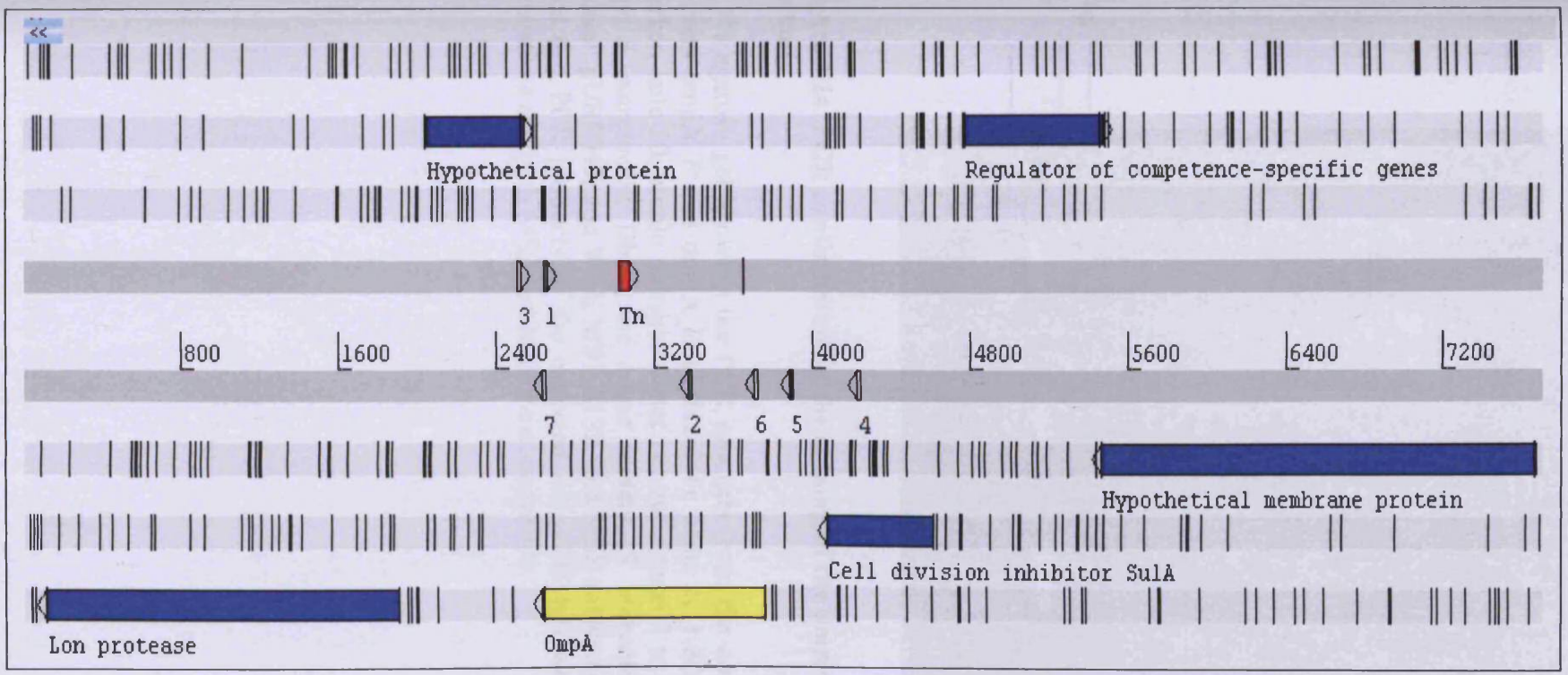


Figure 3.23. Complementation of the 12-F6 (*ompA*) mutant

Artemis screen (929) showing the map of DNA surrounding the disrupted putative outer membrane protein A (OmpA) gene in mutant 12-F6 (in yellow) and the surrounding genes (in blue). The putative protein products are shown under the corresponding ORF. Sites 1 and 2 represent primer ompA_F and ompA_R respectively, used for *ompA* amplification (Table 3.4). Sites 3 and 4 represent primers ompA_F_Eco and ompA_R_Eco respectively, used for the amplification of the *ompA* gene for cloning and complementation (Table 3.4). Sites 5, 6, and 7 represent the promoter region, translation start and stop signals respectively for the *ompA* gene as described by Braun and Cole (95). Tn represents the site of insertion of the mini Tn5Km2 transposon in mutant 12-F6.

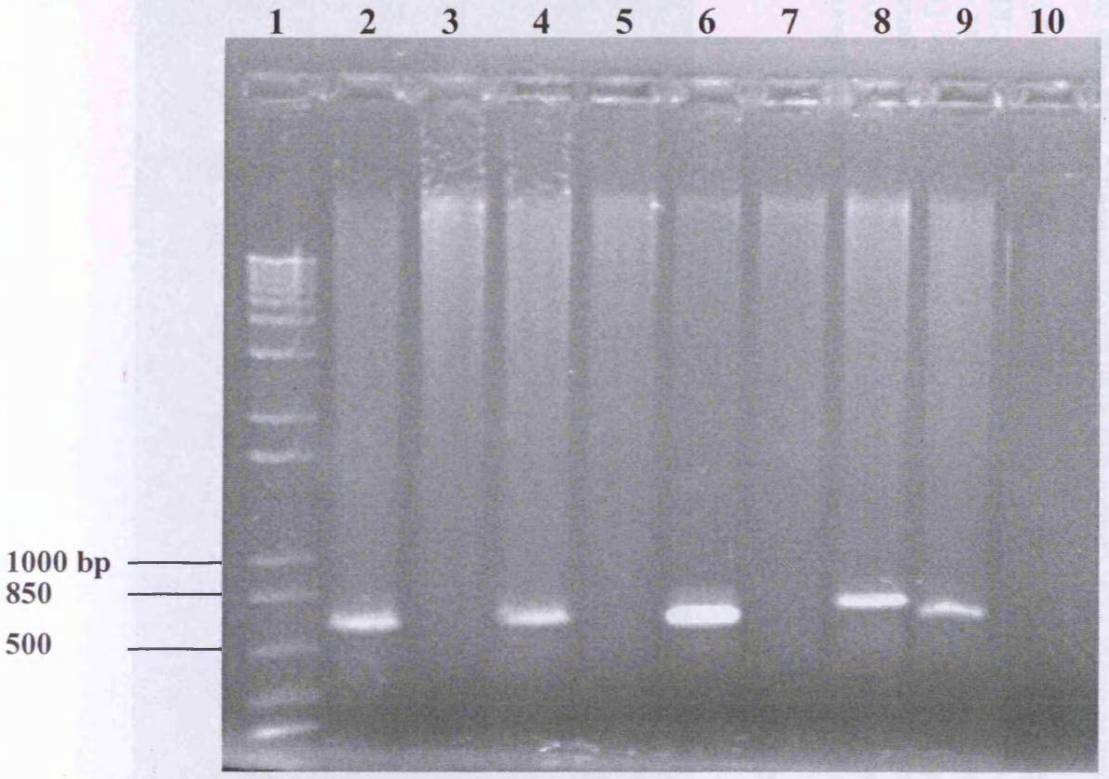


Figure 3.24. PCR confirmation of the cloning of the *ompA* gene into the *ompA* mutant

A 1% agarose gel showing the PCR products from the *ompA* gene amplification using primers ompA_F and ompA_R (Table 3.4). Lane 1; 1-Kb+ DNA ladder used as size marker, lane 10; sterile polished water control. Lanes 2 to 9 represent DNA from, wild type *S. marcescens* Db11, the *ompA* mutant, *S. marcescens* Db11::pURF047, *ompA* mutant::pURF047, and WS1, MS1, MS2, and MS3 transconjugants respectively. It can be seen that PCR products of the expected size (743 bp) were obtained in all wild type derivatives and both MS2 and MS3 transconjugants.

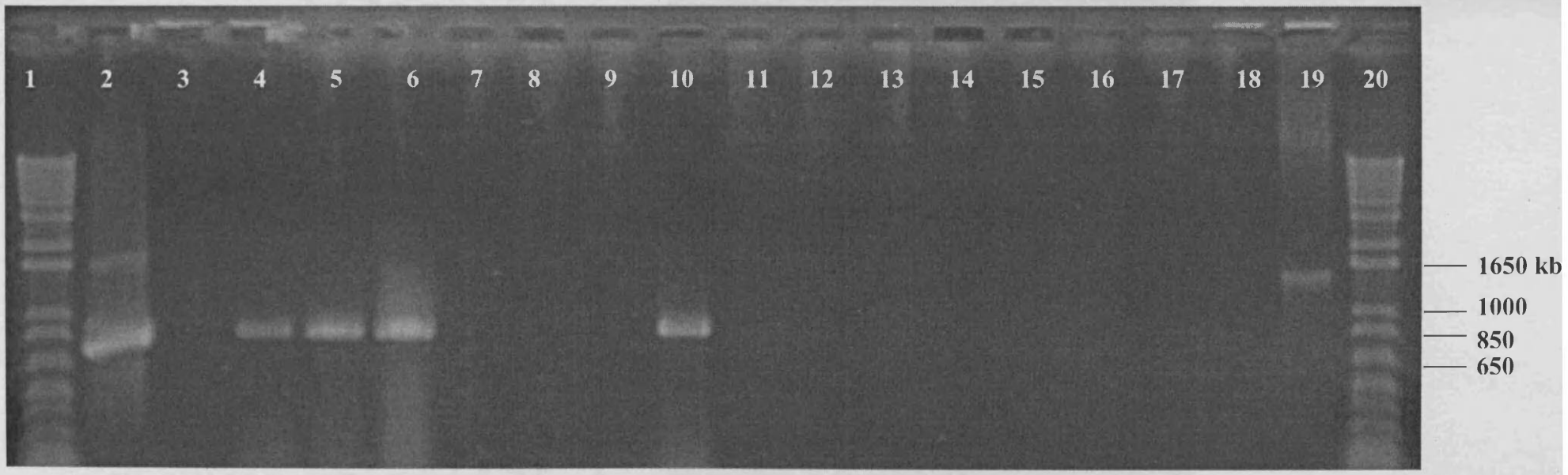


Figure 3.25. The *ompA* reverse transcriptase (RT) polymerase chair reaction (PCR)

A 1% agarose gel showing the products of *ompA* PCR using primers ompA_F and ompA_R (Table 3.4) and cDNA generated by reverse transcriptase reactions using RNA extracted from wild type *S. marcescens* Db11, *ompA* mutant and number of their derivatives. Wild type DNA was used as a positive control for the *ompA* gene (lane 2), Sterile polished water was used as negative control (lane 3). Lanes 4 to 10 represent PCR products using cDNA generated by reverse transcriptase reaction using RNA extracted from: *S. marcescens* Db11, *S. marcescens* Db11::pURF047, WS1 (*S. marcescens* Db11:: SABE*ompA*), the *ompA* mutant, *ompA* mutant::pURF047, and MS2 and MS3 transconjugants (*ompA* mutant:: SABE*ompA*) respectively. Lanes 11 to 17 represents the negative controls for the RT reactions for *S. marcescens* Db11, *S. marcescens* Db11::pURF047, WS1, *ompA* mutant, *ompA* mutant::pURF047, and MS2 and MS3 transconjugants respectively (Table 3.5). Lane 18 represents the RT negative control (Table 3.5), and lane 19 the RT reaction positive control (1.2 Kb kanamycin positive). Lanes 1 and 20 represents 1 Kb+ DNA ladder.
It can be seen that PCR products of the expected size (743 Kb) resulted in both positive *ompA* PCR control and from cDNA generated from wild type *S. marcescens* Db11 and its derivatives. Also PCR product was seen in the reaction which used cDNA from MS3 transconjugant. No PCR products were seen in the *ompA* mutant, *ompA* mutant::pURF047, or MS2 and in all the RT negative control reactions.

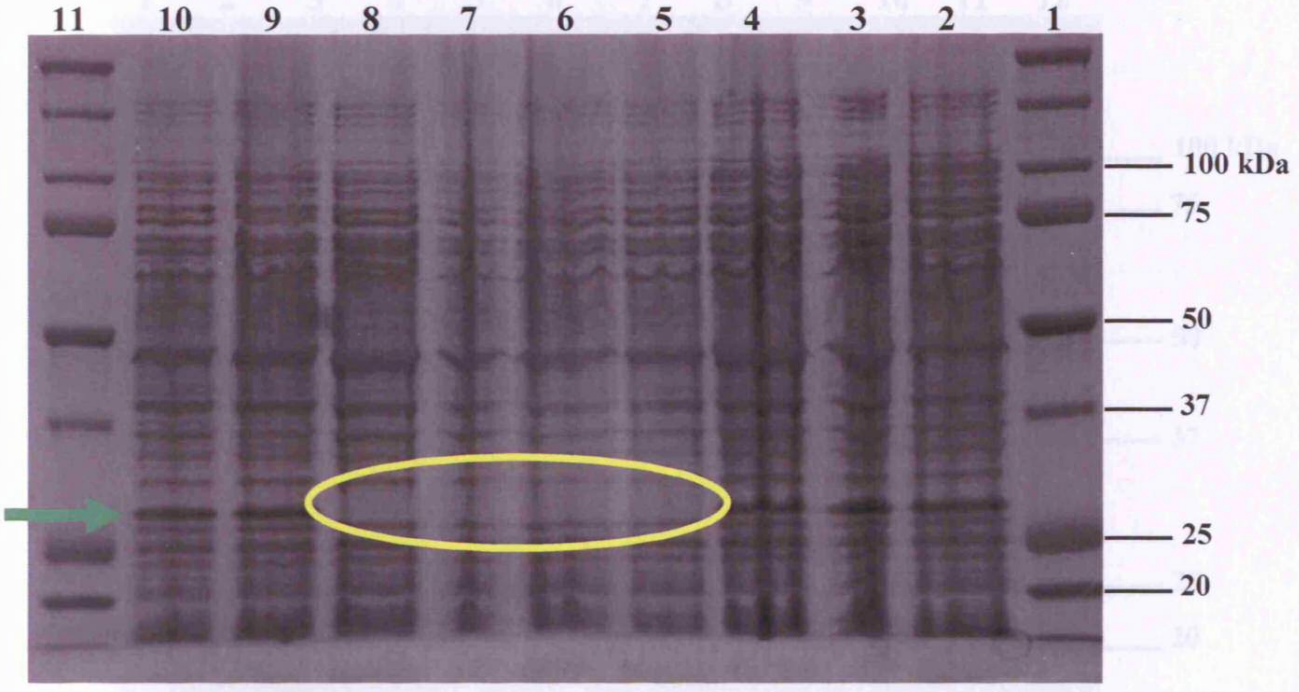


Figure 3.26. The *ompA* mutant whole cell protein electrophoresis

Whole cell protein gel electrophoresis of wild type *S. marcescens* Db11, the *ompA* mutant and their derivatives. Lanes 1 and 11 represents precision Plus dual colour protein standard used as a size marker. Lanes 2, 9 and 10 represent proteins from wild type *S. marcescens* Db11. Lanes 3 to 8 represent proteins from: *S. marcescens* Db11::pURF047, WS1, the *ompA* mutant, *ompA* mutant::pURF047 and MS2 and MS3 transconjugants respectively. It can be seen that the OmpA protein, ≈ 30-kDa (arrow), is present in the wild type and its derivatives but not in the *ompA* mutant or its derivatives (yellow circle). This confirmed that the *ompA* mutant lacks the OmpA protein but also that the OmpA protein failed to be expressed in MS2 and MS3 transconjugants.

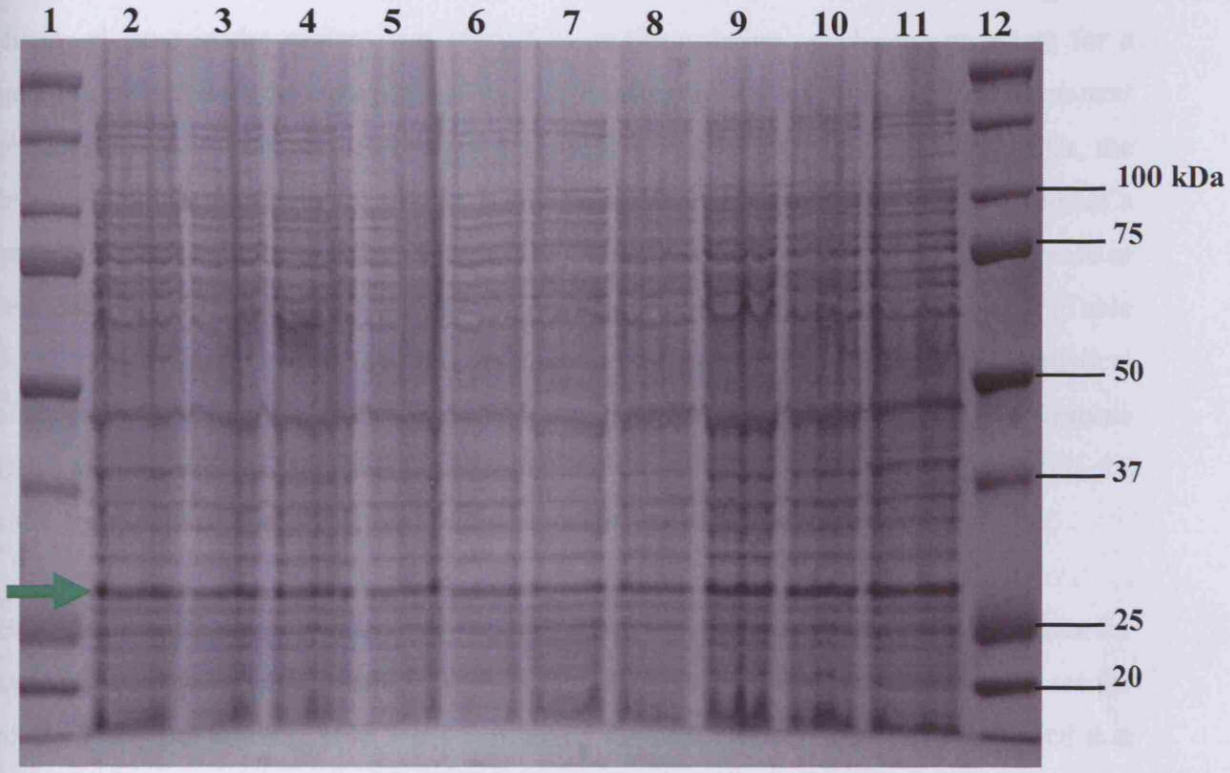


Figure 3.27. Biocide exposure and expression of OmpA in *S. marcescens* Db11

Protein profiles of *S. marcescens* Db11 grown in biocide free TSB (lanes 2 and 11), and biocide treated *S. marcescens* Db11 cultures. Lanes 1 and 12 represent precision Plus dual colour protein standard used as a size marker. Lanes 2, 3, 4, and 5 represent protein profiles from *S. marcescens* Db11 cultures grown in TSB in the presence of 40, 30, 20 and 10 μg/ml triclosan respectively. Lanes 6, 7, 8, and 9 represent protein profiles from *S. marcescens* Db11 cultures grown in TSB in the presence of 14, 8, 4, and 1 μg/ml chlorhexidine diacetate. The OmpA protein is indicated by the arrow. No evidence of increase expression of the OmpA was observed in the biocide-treated cultures.

3.4.15 Detailed analysis of the *ndpA* biocide mutant

Mutant N3-B8 showed increased sensitivity to triclosan, and resistance to cetylpyridinium chloride and chlorhexidine diacetate in the initial biocide screenings. The disrupted gene in the mutant was identified as the putative *ndpA* gene encoding for a putative nucleoid-associated protein in *S. marcescens* Db11 (Table 3.25). The mutant phenotype was investigated further by determining its biocides agar and broth MICs, the biocides lethality effects on the mutant, potassium leakage tests, and the mutant's antibiotic susceptibility profile. On agar, the mutant showed 100% and 44.5% increase in resistance to chlorhexidine diacetate and cetylpyridinium chloride respectively (Table 3.35). It also showed over 15% increase sensitivity to triclosan (Table 3.35). Statistical analysis showed that the differences in MIC values between the wild type *S. marcescens* Db11 and the *ndpA* mutant were significant at 90%, 85% and 70% confidence levels for chlorhexidine diacetate, cetylpyridinium chloride and triclosan respectively.

However, as with the *ompA* mutant, we noted that the change in the agar MIC values for these biocides was reproducible and generally constant in all our replica tests. The results of the agar MIC ratios (Table 3.13) confirmed the above observation. They showed that for chlorhexidine diacetate the ratio values between the mutant and the wild type for the maximum and the minimum MIC values generated in our replica tests were 1.4 and 1.5 respectively. Similarly, the ratio values for the same above parameters were 0.9 and 0.78 for triclosan and 1.83 and 2 for cetylpyridinium chloride. These results showed that although the change in susceptibility of the *ndpA* mutant to cetylpyridinium chloride, triclosan and chlorhexidine diacetate, was not large, it was nevertheless reproducible and genuine. Broth MICs for the *ndpA* mutant were also determined for chlorhexidine diacetate and cetylpyridinium chloride (Table 3.35). The mutant showed at least 172% and 34% increase in resistance to cetylpyridinium chloride and chlorhexidine diacetate respectively. This increase in resistance was statistically significant at the 95% confidence level.

The lethality effect of the four biocides on the *ndpA* mutant was investigated by the suspension (section 3.3.5.3) and the potassium leakage tests (section 3.3.6). Results of the former (Table 3.36) showed that the number of the *ndpA* mutant cells killed after exposure to 50 μg/ml *ortho*-phthalaldehyde for 5 min was not statistically significantly different from that of the wild type. On the other hand, 10 min exposure to 20 μg/ml chlorhexidine diacetate or to 8 μg/ml cetylpyridinium chloride caused nearly 14.5% and 19% respectively less cell death in the mutant compared with the wild type. A 5 min exposure to 4000 μg/ml triclosan lead to 40% more mutant cells killed than it did for the wild type. All the above differences in cell mortality compared with the wild type were statistically significant at the 95% confidence level.

The other parameter used to determine the lethality effect of the above concentrations of biocide on the mutant was to measure the amount of potassium leaked from a biocide-treated suspension of mutant and compare it with the wild type. The results (Table 3.36) showed that exposure to the two biocides, chlorhexidine diacetate and cetylpyridinium chloride, at the above concentrations and exposure time, lead to less potassium to be released from the mutant suspension than the wild type. The decreases in the amount of potassium released were of 20% and 33.6% for cetylpyridinium chloride and chlorhexidine diacetate respectively. A 5 min exposure to 4000 μg/ml triclosan on the other hand, caused 37.5% more potassium to be released in the mutant than it did in the wild type. The changes in the extracellular potassium in the mutants suspensions compared with the wild type were all statistically significant at the 95% confidence level.

The antibiotic susceptibility profile for the *ndpA* mutant was determined for ten antibiotics and compared to that of the wild type. The results (Table 3.37) showed that on the bases of the BSAC MIC breakpoints for *Enterobacteriaceae* (640) the *ndpA* mutant was resistant to the two aminoglycosides; amikacin and tobramycin as well as to chloramphenicol, but sensitive to the two carbapenems; imipenem and meropenem, as well as to antibiotics from other classes including ceftazidime, piperacillin, and ciprofloxacin. When the antibiotic MIC values for the mutant were compared with that of the wild type, the mutant was more sensitive than the wild type to the two carbapenems;

imipenem and meropenem, as well azithromycin and ciprofloxacin. All these changes in MIC values were statistically significant at the 95% confidence level. No changes in MIC values were observed for the mutant with amikacin, tobramycin, chloramphenicol, ceftazidime, piperacillin or (1/19) trimethoprim/sulfamethoxazole. The mutant was also sensitive to 10 μg/ml gentamicin but resistant to 100 μg/ml kamamycin and to 100 units/ml polymyxin B.

3.4.15.1 Complementation of the *ndpA* mutant

3.4.15.1.1 Complementation steps

To confirm that the phenotypic change in the *ndpA* mutant was due to the disruption of the *ndpA* gene in *S. marcescens* Db11, complementation of the mutant was attempted as described in section 3.3.13. It involved the amplification of a wild type *S. marcescens* Db11 DNA fragment containing the *ndpA* gene (Figure 3.28) using primers ndpA_F_Bam, and ndpA_R_Bam (Table 3.4) by epicentre "Failsafe" PCR (section 3.3.13.2). These primers also introduced a unique *Bam*HI restriction sites tails at each end of the PCR product. A genetic construct, SABE*ndpA* was generated by ligation of the above epicentre "Failsafe" PCR product to a *Bam*HI digested pURF047 cloning vector. One Shot OmniMAX^{TM} 2 T1 chemically competent *E. coli* was transformed with the SABE*ompA* construct (section 3.3.13.4), and used in tri-parental matings to introduce the SABE*ndpA* construct into the *ndpA* mutant (section 3.3.13.6). A number of transconjugants including NS9, NS12, NS14, NS15 and NS18 were isolated. Similarly the SABE*ndpA* construct was introduced into the wild type *S. marcescens* Db11 to determine the effect of harbouring an extra copy of the *ndpA* gene on the organism, and four transconjugants WSa, WSb, WSc and WSd were isolated. Moreover, tri-parental mating was used to introduce a copy of the cloning vector pURF047 into the *ndpA* mutant to determine whether the presence of the vector alone could influence the mutant's phenotype.

Successful introduction of the SABE*ndpA* into the transconjugants was checked by extracting the construct from these cells and excising the cloned DNA fragment containing the *ndpA* gene using *Bam*HI digestion. The results were visualised by agarose gel electrophoresis. Results (Figure 3.29) showed that *Bam*HI digestion caused the linearization of the pURF047 vector and the excision of the cloned DNA fragment of the correct size (2739 bp) from two (SWc and SWd) out of the four wild type transconjugants and from four (NS12, NS14, NS15, and NS18) out of the five mutant transconjugants tested. The result confirmed that the *ndpA* gene construct had been successfully introduced into the SWc, SWd, NS12, NS14, NS15, and NS18 transconjugants. Further investigations to determine whether the phenotypic complementation of the *ndpA* mutant was successful (section 3.3.13.7) were then undertaken.

3.4.15.1.2 Phenotypic complementation analysis

The above results confirmed that in transconjugants NS12, NS14, NS15, and NS18, the *ndpA* complementation had been successful in terms of introducing a wild-type copy of the gene into the mutant. To investigate further, phenotypic complementation analysis was performed on transconjugants to check for restoration of the wild type phenotype. The study also included wild type transconjugants, as well as wild type and *ndpA* mutant containing the pURF047 vector. No noticeable difference in growth was seen in the *ndpA* mutant cells containing the pURF047 vector or in the transconjugants compared to the wild type *S. marcescens* Db11. This suggests that the presence of the pURF047 vector or the introduced copy of the *ndpA* gene in the cells did not affect their growth fitness. The results of agar biocide MICs on the NS12, NS14, NS15, and NS18 transconjugants showed no significant difference in their MIC values from those of the wild type. For instance, for cetylpyridinium chloride, growth of the wild type, the wild type transconjugants and the *ndpA* mutant transconjugants was inhibited at concentration of 100 µg/ml, while the *ndpA* mutant grew at a concentration of 240 µg/ml. These agar MIC results meant that there was restoration of the wild type phenotype in the NS12, NS14, NS15, and NS18 transconjugants. Moreover the results also showed that the presence of

the pURF047 vector in both the wild type and the *ndpA* mutant or the introduced extra copy of the *ndpA* in the wild type had not affected biocide susceptibility.

Table 3.35. Agar and broth MICs for both wild type *S. marcescens* Db11 and the *ndpA* mutant.

Biocide	Broth (TSB) MIC µg/ml (± SD)		Agar (TSA) MIC µg/ml (± SD)	
	***S. marcescens* Db11**	***ndpA* mutant**	***S. marcescens* Db11**	***ndpA* mutant**
CPC	5.5 (0.70)	>15	100 (28.3)	200 ± (28.3)
CHX	7.5 (0.70)	>10	18 (2.83)	26 ± (2.83)
TRI	Nd	Nd	3900 (141)	3300 ± (424)
OPA	Nd	Nd	3000 (283)	3000 (283)

SD; standard deviation, TRI; triclosan, OPA; *ortho*-phthalaldehyde, CPC; cetylpyridinium chloride, CHX; chlorhexidine diacetate, Nd; not determined.
Only values that were statistically significantly different at 95% confidence from those of the wild type are highlighted (red; statistically lower, blue; statistically higher).

Table 3.36. Comparison between *S. marcescens* Db11 and the *ndpA* mutant using two biocide lethality measuring parameters.

Biocide	Suspension tests [Log_{10} reduction in cfu/ml]		Potassium leakage tests [Potassium released (ppm)]	
	***S. marcescens* Db11**	***ndpA* mutant**	***S. marcescens* Db11**	***ndpA* mutant**
CPC	3.13	2.55	0.10	0.08
CHX	2.57	2.20	1.07	0.71
TRI	3.11	4.33	0.80	1.10
OPA	1.18	1.09	0.50	0.50

TRI; triclosan at 4000 µg/ml after 5 min exposure, OPA; *ortho*-phthalaldehyde at 50 µg/ml after 5 min exposure, CPC; cetylpyridinium chloride at 8 µg/ml after 10 min exposure, CHX; chlorhexidine diacetate at 20 µg/ml after 10 min exposure. Only values that were statistically significantly different at 95% confidence from those of the wild type are highlighted (red; statistically higher, blue; statistically lower).

Table 3.37. Antibiotic MICs for the wild type *S. marcescens* Db11 and the *ndpA* mutant.

Antibiotic	MIC (μg/ml)		Mutant sensitivity	
	S. marcescens Db11	*ndpA* mutant	Compared to BSAC MIC breakpoints*	Compared to *S. marcescens* Db11
Aminoglycosides				
Amikacin	8	8	R	Nc
Tobramycin	25.1	32	R	Nc
Cephalosporins				
Ceftazidime	0.71	0.5	S	Nc
Carbapenems				
Imipenem	0.59	0.25	S	S
Meropenem	0.12	0.064	S	S
Macrolides				
Azithromycin	>256	96	-	S
Penicillins				
Piperacillin	2	2	S	Nc
Quinolones				
Ciprofloxacin	0.17	0.125	S	S
Sulfonamides**				
Trimethoprim/ sulfamethoxazole	0.65	1	-	Nc
Others				
Chloramphenicol	24	24	R	Nc

Statistically significant increases in susceptibility (red) are highlighted
* Susceptibility based on BSAC MIC breakpoints for *Enterobacteriaceae* (640).
** Trimethoprim/sulfamethoxazole (1/19).
R; resistant, S; susceptible, Nc; no change in sensitivity

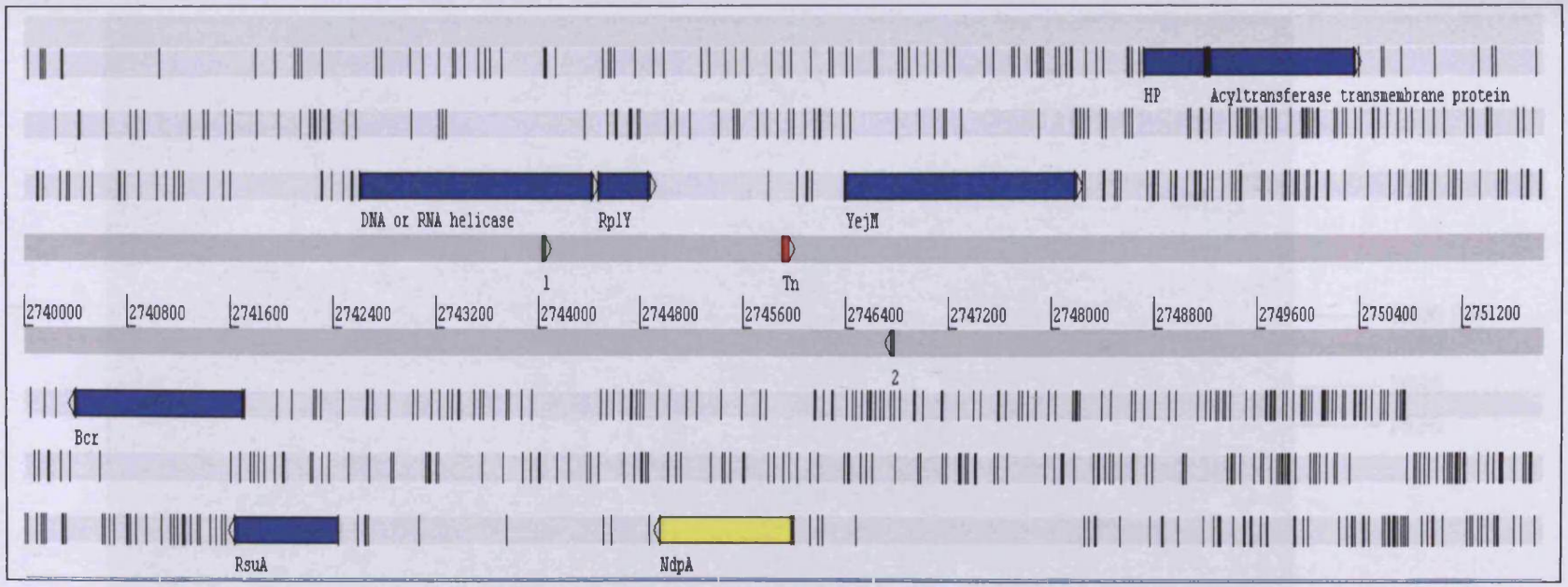


Figure 3.28. Complementation of the N3-B8 (*ndpA*) mutant

Artemis screen (929) showing map of the DNA surrounding the disrupted putative nucleoid-associated protein (NdpA) gene in mutant N3-B8 (in yellow) and the surrounding genes (in blue). The putative protein products are shown under the corresponding ORF. Sites 1 and 2 represent primer ndpA_F_Bam and ndpA_R_Bam respectively, used for the amplification of the *ndpA* gene for cloning and complementation (Table 3.4). Tn represents the site of insertion on the mini Tn5Km2 transposon in mutant N3-D8. Bcr; bicyclomycin resistance protein, RsuA; ribosomal small subunit pseudouridine synthase A, RplY; 50S ribosomal protein L25, NdpA; nucleoid-associated protein, YejM; sulphatase, HP; hypothetical protein HS_1396.

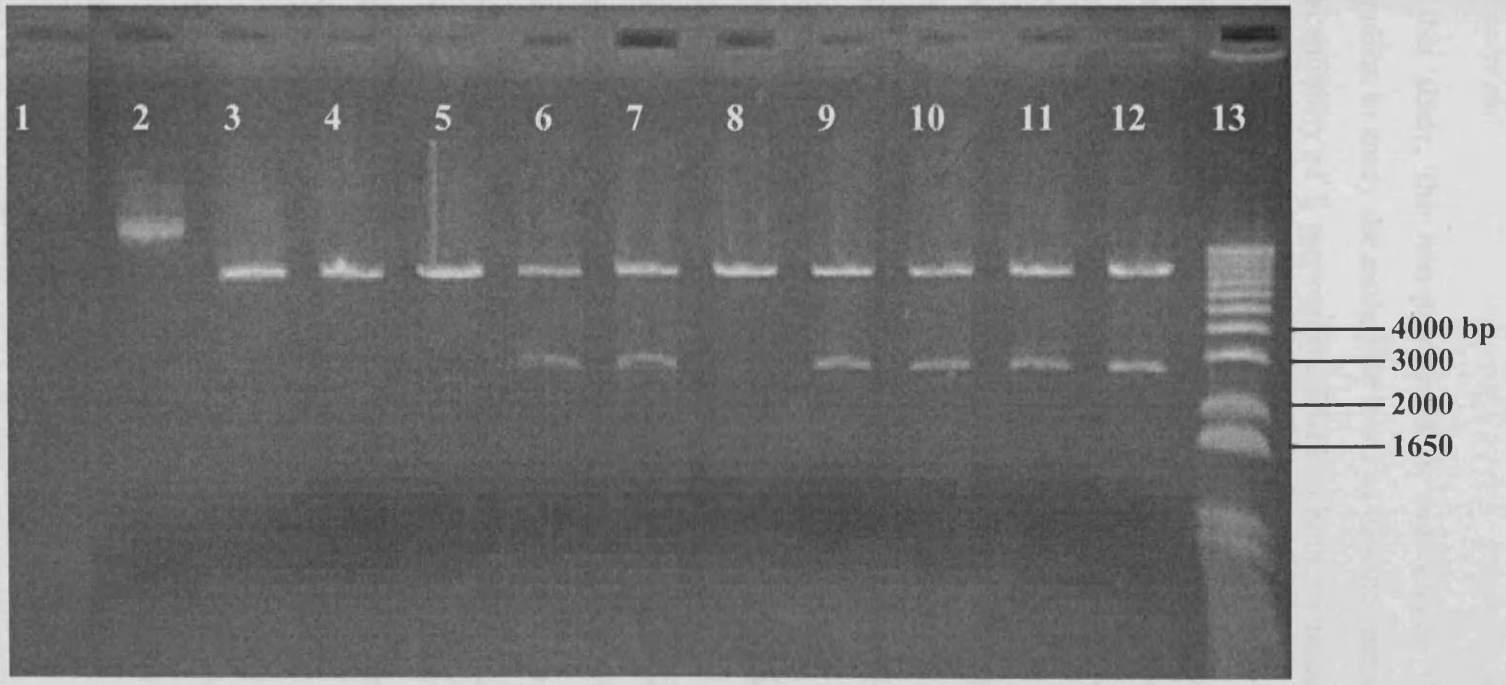


Figure 3.29. Confirmation of the cloning of the *ndpA* gene into the *ndpA* mutant

A 1% agarose gel showing confirmation of cloning of the *S. marcescens* Db11 *ndpA* gene into the *ndpA* mutant. Plasmids were extracted from both wild type and *ndpA* mutant transconjugants and cut with *Bam*HI to excise the cloned fragment of DNA containing the wild type *ndpA* gene. Lane 1; negative control, lane 13; 1-Kb+ DNA ladder used as size marker, lane 2; uncut pURF047, lane 3; pURF047 cut with *Bam*HI. Lanes 4, 5, 6, and 7 represent wild type transcongugants (*S. marcescens* Db11:: SABE*ndpA*) WSa, WSb, WSc, and WSd respectively. Lanes 8, 9, 10, 11, 12 represent *ndpA* mutant transconjugants (*ndpA* mutant::SABE*ndpA*) NS9, NS12, NS14, NS15 and NS18 respectively. Digestion of the SABE*ndpA* construct with *Bam*HI would excise the cloned DNA fragment containing the *ndpA* gene and linearize the pURF047 vector. It can be seen that this was the case with transconjugants SWc, SWd, NS12, NS14, NS15, and NS18, where the excised DNA fragments of the expected size (2739 bp) were observed along with the linearized pURF047.

3.5 DISCUSSION

3.5.1 *S. marcescens* Db11 and antimicrobial agents

In this study, the non-pigmented *S. marcescens* Db11 was chosen as a model organism to study the molecular basis of biocide resistance and sensitivity in *Serratia*. Susceptibility of *S. marcescens* Db11 to both the inhibitory and killing effects of four biocides (triclosan, chlorhexidine diacetate, cetylpyridinium chloride, and *ortho*-phthalaldehyde), and its antibiotic profile for 10 agents (amikacin, azithromycin, chloramphenicol, ciprofloxacin, tobramycin, trimethoprim/sulfamethoxazole, meropenem, ceftazidime, imipenem, and piperacillin) were determined.

The two surface-active agents chlorhexidine diacetate and cetylpyridium chloride were the most effective at inhibiting the growth of *S. marcescens* Db11 with agar MIC values of 18 μg/ml and 100 μg/ml respectively. *Ortho*-phthalaldehyde and triclosan were less effective and had higher agar MIC values of 3000 μg/ml and 3900 μg/ml respectively. The large difference in MIC values between the latter two agents and those of the two surface active agents could be due to interaction between the agar and the biocides, which in the case of triclosan and *ortho*-phthalaldehyde could play a role in affecting their antimicrobial action. Interaction between biocides and agar appears to also affect the growth inhibitory action of both surface active agents. However, this effect seems to be less significant in relation to chlorhexidine diacetate, as it had a broth MIC value of 7.5 μg/ml compared to 18 μg/ml on agar. From MIC values, cetylpyridium chloride on the other hand, is nearly 20 times more effective at inhibiting growth of *S. marcescens* Db11 in broth than it is on agar.

Evidence of the biocides lethal effect on the *S. marcescens* Db11 cells was observed from both the killing and the potassium leakage experiments, where two killing patterns were identified. The first was in relation to the surface active agents, cetylpyridium chloride and chlorhexidine diacetate, where the amount of killing increased with time of exposure to the biocide. The other pattern was in relation to triclosan and to a lesser extent to *ortho*-phthalaldehyde, where most of the killing occurred in the first 5 min of exposure, after that there was no significant change in

the killing effect of the two biocides. Lethal effects of chlorhexidine diacetate and cetylpyridinium chloride were measured at levels much lower than their "in-use" concentrations. The concentrations tested for triclosan and *ortho*-phthalaldehyde were comparable to those used in practice. Based on the killing tests results, the chlorhexidine diacetate and cetylpyridinium chloride "in-use" concentrations are effective at reducing the total *S. marcescens* Db11 cell counts, whereas those of triclosan and *ortho*-phthalaldehyde may not be.

S. marcescens is naturally resistant to many antibiotics including penicillins, cephalosporins, and polymyxins, and is able to rapidly acquire resistance to other agents (section 3.1.5). Although resistant strains have been reported (192), aminoglycosides have good activity against *S. marcescens*. In a study by Stock *et al.* (1022) on 77 *S. marcescens* isolates, they found that 100% of strains tested were susceptible to amikacin, and 80% showed intermediate or susceptible phenotype to tobramycin. All strains were susceptible to the β-lactams, ceftazidime, imipenem, and meropenem and 75% were sensitive to piperacillin. The percentages of isolates susceptible to the quinolones ciprofloxacin and to the sulphonamide trimethoprim/sulfamethoxazole were 100% and 83% respectively. It was also reported that the majority of the strains (90%) were resistant to the macrolide azithromycin and that 75% of the isolates were also resistant to chloramphenicol. Comparison between the above and the antibiotic susceptibility results for *S. marcescens* Db11 (based on E-test MIC values and the BSAC MIC breakpoints for *Enterobacteriaceae* (640)), in this study shows some contradictions. In this study *S. marcescens* Db11 was resistant to both aminoglycosides used (amikacin and tobramycin) as well as to chloramphenicol. It was however, sensitive to all other agents tested including to representative from the β-lactam family.

The natural cell impermeability of *S. marcescens* and its production of antibiotic inactivating enzymes could explain the observed response of the organism to aminoglycosides. As the MIC value for amikacin (8 μg/ml) was on the upper limit of the intermediate susceptible phenotype according to the BSAC MIC breakpoints, this low level resistance could be explain by the natural cell impermeability of *S. marcescens* and by the possibility of low level expression of acetyltransferase AAC(6′) which is active against amikacin (section 3.1.5.1.2). The same mechanisms

could be involved in tobramycin resistance in strain Db11, although the high level of resistance observed in this strain suggests that other factors may be involved. The production of the other known *S. marcescens* aminoglycoside inactivating enzyme, acetyltransferase AAC(3)-I which is active against gentamicin (section 3.1.5.1.2), is unlikely as strain Db11 was sensitive to 10 μg/ml gentamicin.

In comparison to the Gram-positive *S. aureus*, *S. marcescens* Db11 is more resistant to antimicrobial compounds. This becomes apparent when the agar MIC values of agents are compared for the two species. Antibiotics MIC values of the *S. aureus* reference strains NCTC 6571, ATCC 25923 and ATCC 29213 (17) show that for amikacin, tobramycin, azithromycin, chloramphenicol, imipenem and meropenem, *S. marcescens* Db11 is more resistant than *S. aureus*. While this comparison is by no means accurate, and does not relate to the wide range of antibiotic resistant *S. aureus* strains, it is indicative that *Serratia* is generally more resistant to antibiotics than Gram-positives such as *S. aureus*.

The same observations can be made for biocides, where for example in a study by Seaman *et al.* (965) the MIC value of triclosan for a number of *S. aureus* strains was reported to be 0.063 μg/ml. Similarly in a study of triclosan resistance in *S. aureus*, which including several MRSA strains, Suller and Russell (1031) reported that triclosan MIC values for the strains studied ranged from 0.025 μg/ml to 1 μg/ml. The control strain NCTC 6571 had an MIC value of 0.025 μg/ml. Comparison between triclosan MIC values for *S. aureus* NCTC 6571 and *S. marcescens* Db11 shows that *S. marcescens* Db11 is 160000 times more resistance to triclosan than *S. aureus* NCTC 6571. Differences in susceptibility to other biocides are also apparent. Chlorhexidine MIC values for *S. aureus* reported by Wallhausser (1116) were 0.5-1 μg/ml, and Yamamoto *et al.*(1176) reported them to be 1.56-6.25 μg/ml in the strains they studied, with the MRSA strain O_3 being most resistant. Seaman *et al.* (965) reported chlorhexidine MIC values of 1-3 μg/ml for the *S. aureus* strains they studied and in a study by Irizarry *et al.* (479) they found that MIC values for chlorhexidine did not exceed 2 μg/ml in 83% of methicillin-sensitive *S. aureus*. Although antibiotic resistant *S. aureus* such as MRSA were shown to be more resistant to chlorhexidine than sensitive strains (479), the above still shows that *S. aureus* is much more sensitive to this agent than *S. marcescens* Db11, which had an MIC of 18 μg/ml.

Similar observations can be made for the QAC, cetylpyridinium chloride. Seaman *et al.* (479) reported MIC values for the agent to be 1-3 μg/ml in all *S. aureus* strains they tested. Irizarry *et al.* found that as for chlorhexidine, the majority (93%) of methicillin-sensitive *S. aureus* tested had MIC value for cetylpyridinium chloride equal or lower than 2 μg/ml. The study also showed that MRSA have increased MIC to cetylpyridinium chloride compared to the sensitive strains (479), an observation also reported by Suller and Russell (1030) who found MRSA to have MIC values for the QAC 2- to 4-fold higher than sensitive strains. Nevertheless, the above reports show that compared with *S. marcescens* Db11 with a cetylpyridinium chloride MIC of 100 μg/ml, *S. aureus* is much more sensitive to the agent than the *Serratia* strain.

Serratia resistance to antimicrobial agents is not only apparent in comparison to Gram-positive bacteria but also to many Gram-negatives, including very close relatives in the *Enterobacteriaceae* family such as *E. coli*. In fact the levels of *S. marcescens* resistance are comparable to those of *P. aeruginosa*, which shows high intrinsic resistance to many antimicrobial agents (157). In this study, *S. marcescens* Db11 was more resistant than the *E. coli* NCTC 1048 reference strain for nearly all antibiotics tested. Similar observations are seen if the antibiotics MICs of *S. marcescens* Db11 calculated in this study were to be compared to those of other *E. coli* reference strains such as NCTC 10418 and ATCC 25922 (17). In fact, for many antibiotics, the MIC values of *S. marcescens* Db11 are comparable to those reported for *P. aeruginosa* reference strains NCTC 10662 and ATCC 27853 (17). Strain Db11 is even more resistant than the above two *P. aeruginosa* strains for the aminoglycosides amikacin and tobramycin.

The level of antimicrobial resistance in *S. marcescens* Db11 compared to other Gram-negatives extends to biocides. Results of the biocides MICs determination and both the killing and potassium leakage experiments showed that *S. marcescens* Db11 is more resistant than *E. coli* NCTC 1048 to both the inhibitory and killing effects of the biocides tested. From agar MICs, *E. coli* NCTC 1048 was 39000 times more sensitive to triclosan than *S. marcescens* Db11. The triclosan MIC value of the latter is comparable to that of *P. aeruginosa* PAO1 which was shown to resist triclosan concentrations higher than 1000 μg/ml due to its efflux mechanisms (167). The *P.*

aeruginosa chlorhexidine MIC of 5-60 μg/ml reported by Wallhausser (1116) is also comparable to that of *S. marcescens* Db11.

Large aspects of the tolerance of *S. marcescens* Db11 to antimicrobial agents compared to Gram-positives such as *S. aureus* or even to close relatives such as *E. coli* could be explained in relation to its membrane permeability, porins and efflux systems. As a Gram-negative, *S. marcescens* Db11 has an extra outer membrane which Gram-positives such as *S. aureus* do not have. This membrane provides an extra permeability barrier which significantly limits the uptake of antimicrobial agents by the cells. Of importance in this context are the lipopolysaccharides and outer membrane proteins of the cell which have been discussed in section 1.5.1.1. For instance, the characteristic resistance of *S. marcescens* and *Proteus* species to polymyxins B and E and to the polycationic antimicrobial proteins found in granulocyte granules (1094) in comparison to other enteric bacteria, has been related to differences in the lipopolysaccharide core composition between these two bacteria and *E. coli* K-12 and *S. typhimurium* (848).

In conclusion, *S. marcescens* Db11 has been shown to be more affected by surface-active agents such as chlorhexidine and cetylpyridium chloride than by the phenolic compound triclosan or the aldehyde *ortho*-phthalaldehyde. This strain was interesting in that unlike many other reported *S. marcescens* strains, it was resistant to the aminoglycosides and sensitive to β-lactams. Although it is not possible to arrive at a generalised conclusion from the study of a single strain, nevertheless, results from this study and reports in the literature demonstrate that *S. marcescens* is relatively resistant to antibiotics and biocides compared to the Gram-positive *S. aureus* and to its close relatives in the *Enterobacteriaceae* family such as *E. coli*. It is clear that the outer membrane of *S. marcescens* represents an important permeability barrier protecting it from the effect of antimicrobial agents. The organism contains a number of multidrug efflux pumps and porins, which along with its other outer membrane components has an impact of its antimicrobial susceptibility. The organism can also produce and secrete a number of proteins and enzymes which are able to degrade or inactivate antimicrobial agents (section 3.1.5). We can speculate that the relative resistance to antimicrobial agents seen in *S. marcescens* Db11 can be at least in part a result of its outer membrane characteristics.

3.5.2 *S. marcescens* Db11 mutants

3.5.2.1 Overview

The study set out to (i) identify the molecular basis of biocide susceptibility and resistance in the Gram-negative *S. marcescens* strain Db11, and to (ii) determine if there is a relationship between biocides and antibiotic susceptibility in this organism. Random transposon mutagenesis using the mini-Tn*5*Km2 transposon was used to generate 26 *S. marcescens* Db11 mutants with altered biocide sensitivity compared with the wild type. These mutants along with two control mutants were all confirmed to be *S. marcescens* Db11 by RAPD analysis and their specific growth rate values demonstrated that the majority of them were as growth fit as the wild type.

Biocide susceptibility patterns were determined by investigating both the growth inhibitory and killing effects of the four biocides on the mutants. Biocides agar MICs demonstrated that changes in biocides susceptibility of the mutants tested were relatively small. However, these changes were reproducible. The results also showed that 84% of the mutants tested had increase in sensitivity or resistance to more than one biocide. The most dominant sensitivity was to cetylpyridium chloride whereas resistance to chlorhexidine diacetate was the most common resistance phenotype. Out of the 26 biocide mutants investigated, the percentage of mutants sensitive to chlorhexidine diacetate, cetylpyridium chloride, triclosan and *ortho*-phthalaldehyde were 30%, 53%, 42% and 42% respectively. The proportion of resistant mutants was 23% for chlorhexidine diacetate, 11% for cetylpyridium chloride and *ortho*-phthalaldehyde, and 7% for triclosan. Antibiotic susceptibility profiles for all mutants were determined and revealed that with the exception of one control mutant, all remaining mutants had at least two or more changes in their antibiotic susceptibility profiles compared with the wild type. The results showed that 90% of the mutants had increased susceptibility to at least one antibiotic, and an interesting high percentage (70%) demonstrated increased resistance to at least one antibiotic as well.

The locations of transposon insertion in all but three of the mutants were determined, and 14 putative genes coding for putative proteins with varying functions were found to be disrupted. These functions included anabolism and catabolism, gene regulation,

cell envelope biosynthesis, porin, energy production, and virulence. Multiple insertions into the same genes were also observed in 21% of the disrupted gene, and one putative gene coding for a putative DeoR family transcriptional regulator, had transposon insertion in nine of the mutants sequenced. One possible explanation for the mini-Tn*5*Km2 multiple insertions into the same gene could be due to the gene being an "insertion hotspot" for the transposon, whereby the latter inserts into the gene preferentially. There is evidence for and against this possibility. Codling *et al.* (179) reported the single random insertion of the mini-Tn*5*Km2 transposon into the *S. marcescens* genome with the complete loss of the delivery vector after transposon insertion. However, Payne (807) noted multiple insertions of the mini-Tn*5*Km2 into the same genes in some *B. vietnamiensis* G4 transposon mutants.

3.5.2.2 Transposon-disrupted genes in *S. marcescens* Db11 biocide mutants

3.5.2.2.1 Putative ribonucleotide-diphosphate reductase alpha subunit gene (*nrdA*)

Transposon insertion in mutant N5-B6 was traced to a *nrdA* gene coding for a putative ribonucleotide-diphosphate reductase α subunit. The N5-B6 mutant showed increased sensitivity to triclosan and cetylpyridinium chloride on agar as well as increased susceptibility to meropenem, imipenem, piperacillin, and amikacin. The mutant was however more resistant to ciprofloxacin than the wild type. Ribonucleoside-diphosphate reductase α subunit is part of the ribonucleotide reductases multisubunit enzyme responsible for the reduction of ribonucleotides to their corresponding deoxyribonucleotides, thus generates all precursors for DNA synthesis intended for DNA replication and repair (505, 554). Ribonucleotide reduction occupies a central role in the regulation of the pool sizes of the four dNTPs required for DNA synthesis, even though deoxynucleoside kinases and nucleotidases are also important (554).

The first ribonucleotide reductase was reported in in *E. coli* (864) followed by another in *Lactobacillus leichmannii* (81) a few years later. Nowadays a number of ribonucleotide reductases are known and are grouped into different classes depending on the metal cofactors for their catalytic activity (863, 1024). Many organisms have more than one class of ribonucleotide reductases present in their genomes. Class I

enzymes contain a diiron-oxygen cluster and are found in some bacteria and viruses and in practically all eukaryotic organisms, from yeast and algae to plants and mammals (442, 504). Class II ribonucleotide reductases, found in bacteria and bacteriophages, use coenzyme B12 as the cofactor for their activity. Class III enzymes, found in anaerobic bacteria, bacteriophages, and archaea, use a 4Fe–4S iron–sulfur cluster coupled to S-adenosylmethionine to generate activity. Although the three different classes of ribonucleotide reductase enzymes depend on different metal cofactors for the catalytic activity, the functional and structural similarities suggest that the present-day ribonucleotide reductases have all evolved from a common ancestral reductase (554).

It was reported that ribonucleotide reductase from *E. coli* could be purified into two inactive subunitis, proteins B1 and B2, which when mixed, enzyme activity was readily generated (117, 118). It is now known that in *Enterobacteria* the enzyme's two subunits R1 and R2 proteins are encoded by the *nrdA* and *nrdB* genes respectively. The genes constitute a tightly regulated transcriptional unit where transcription is coupled to the cell cycle and is increased by DNA damage (140, 503, 1032). Gibert *et al.* (337) found that induction of the SOS response in *E. coli* enhanced the expression of the *nrdA* and *nrdB* genes. The mammalian ribonucleotide reductase is also composed of two different dimeric proteins (Rl and R2), and in mouse the genes coding for these proteins (*nrdA*, and *nrdB*) are regulated separately and are located on separate chromosomes (505).

The importance of ribonucleotide reductase in the mechanisms of cell proliferation and DNA repair (471, 1164), and the observation that expression of the enzyme is altered in malignant cells (1131), have made it a target in chemotherapeutic strategies (972, 1131). The genetic changes underlying cancer conversion and progression are accompanied by a decrease in genomic stability of cells (172, 1158) which leads to heterogeneity of tumor cell populations, alterations in response to chemotherapy, and increased malignant potential. For instance Huang *et al.* (455) found that altered expression of the R2 component of the ribonucleotide reductase enzyme is capable of significantly modifying drug sensitivity properties of tumor cells. Because of the key role ribonucleotide reductase play in mammalian cells, the enzyme is viewed as an important target for anticancer and antivirus agents, and several different classes of

ribonucleotide reductase inhibitors have been generated and studied, including triapine, gemcitabine, cytarabine, cladribine and many more (972).

The important role of ribonucleotide reductases in bacteria suggests that disruption of the *nrdA* gene in *S. marcescens* Db11 would have an effect on the function of ribonucleotide reductase protein. Many antimicrobial agents exert their effect by interfering with DNA synthesis. For instance quinolones are known to act by blocking DNA synthesis via inhibition of DNA gyrase needed for this process. Biocides such as acridines dyes act by binding to the double stranded DNA by intercalation between adjacent bases on the same strand blocking replication and transcription (577). Alkylating agents such as ethylene oxide and formaldehyde, affect nucleic acids because of their interaction with the amino groups on the purine and pyrimidine bases (622). Modification of purine and pyrimidine bases is also seen in the action of the vapour-phase disinfectant, ozone (362).

Moreover, biocides such as triclosan and cetylpyridinium chloride, especially at higher concentrations have been shown to affect multiple targets within the cell. Hence it can be speculated that in a NrdA-deficient mutant, where DNA synthesis and repair are affected, the cell is more sensitive to antimicrobial action. If a biocide causes DNA damage in such a mutant, repair of the damaged DNA would not be as efficient as in the wild type, making the mutant more susceptible to the biocide. This could explain the increase in sensitivity of the NrdA-deficient mutant N5-B6 to triclosan and cetylpyridinium chloride. The mutant also became more resistance to ciprofloxacin compared with the wild type. Ciprofloxacin is known to act by interfering with DNA gyrase, hence inhibiting DNA synthesis. It can be speculated that in a mutant where DNA synthesis and repair are inefficient, the actions of a drug that acts on such processes would be inefficient as well. This would make the mutant more resistant to such a drug. Another speculation is related to the putative DNA gyrase subunit A gene located in the vicinity of *nrdA*. Transposon insertion in *nrdA* could have had a polar effect on the expression of the DNA gyrase subunit A gene, leading to increased resistance to ciprofloxacin, which has DNA gyrase as a target. Further studies including complementation of the *nrdA* mutant are needed to investigate the above speculations

3.5.2.2.2 Putative chaperonin genes (*groES* and *groEL*)

The site of the transposon insertion in mutant N5-G6 was located at the junction between the putative *groES* and putative *groEL* genes. The location of the insertion meant that the transposon was able to disrupt one if not both of these genes. On agar, the mutant showed increased sensitivity to both triclosan and *ortho*-phthalaldehyde and was resistant to chlorhexidine diacetate. The antibiotic susceptibility profile for mutant N5-G6 showed that it had increased resistance to meropenem, imipenem, chloramphenicol and piperacillin. It was at the same time more sensitive than the wild type to the two aminoglycosides tested as well as to trimethoprim/sulfamethoxazole.

GroEL belongs to the chaperonin family of molecular chaperones required for the proper folding of many proteins in bacteria. To function properly it requires a co-chaperonin complex GroES. In eukaryotes the heat shock proteins Hsp60 and Hsp10 are structurally and functionally nearly identical to GroEL and GroES respectively. Chaperonins are ubiquitous, essential multisubunit ATPases, in which the subunits form a cylindrical structure (a ring) enclosing a central cavity. They are thought to assist the folding of their target proteins by providing a sequestered environment conducive to correct folding in which extended proteins can fold while shielded from nonproductive interactions with other proteins (128, 403, 981, 1170). Two classes of chaperonin have been defined on the basis of sequence relationships and the requirement for cochaperonin (545). Those which function in conjunction with a cochaperonin such as GroEL, belong to class I chaperonins found in prokaryotes and the organelles descended from them. Chaperonins which do not require a cochaperonin are found in archaebacteria and in the cytosol of eukaryotes, and belong to class II. Only one kind of chaperonin molecule generally exists in any given cellular compartment, therefore, any chaperonin must be capable of facilitating the correct folding of a range of target proteins (757).

In *E. coli*, GroEL with the aid of GroES, encapsulates non-native substrate proteins inside the cavity of the GroEL-ES complex and promotes folding by using energy derived from ATP hydrolysis. GroEL is thought to act by first binding partially folded or misfolded proteins in its central cavity, thus preventing their aggregation (174, 194). The second phase of action involves the central cavity of GroEL, where the

isolated protein folding intermediate is actively folded, after being unfolded, if necessary, to states more committed toward correct folding (1136, 1169). The actions of GroEL and other chaperonins is therefore essential for the folding of proteins in the cell, as well as ensuring the correct expression of genes by correcting the inevitable and potentially irreversible mistakes in protein folding generated from errors in transcription and translation processes (981). In *E.* coli, chaperonin GroEL (together with its cochaperonin GroES) is thought to participate in the facilitated folding of 2 to 7% of newly synthesized proteins (267, 621), and the complex is considered to be a major component of cellular machinery for refolding misfolded cytosolic proteins (1122).

Whether GroEL and/or GroES are directly involved in bacterial resistance and susceptibility to antimicrobial agents is not clear. Interestingly however, Hallett *et al.* (393) found that *E. coli* cells resistant to DNA gyrase inhibitor, ciprofloxacin, overproduced a 60-kDa protein homologue to GroEL. Moreover, as mentioned above the proteins Hsp60 and Hsp10 are structurally and functionally nearly identical to GroEL and GroES. Heat shock proteins are known to be induced under different stress conditions. Ramos *et al.* (853) reported that different subsets of heat shock proteins are induced by different stress conditions in bacteria. Induction of the aromatic degradation pathway in *P. putida* has been shown to involve heat shock response (660). In addition Cudic *et al.* (202) demonstrated that proline-rich cationic antibacterial peptides such as pyrrhocoricin killed β-lactam-, tetracycline- or aminoglycoside-resistant strains of *E. coli*, *S. typhimurium*, *K. pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and even *P. aeruginosa*, by binding to the 70-kDa heat shock protein DnaK and inhibiting protein folding. Chen *et al.* (148) found that heat shock proteins in *S. aureus* were induced 5.1-fold following treatment with triclosan. Moreover, Rees (860) reported that a chlorhexidine resistant mutant of *B. cenocepacia* had a disruption in a gene coding for a heat-shock-like protein.

It is clear that a mutant deficient in either GroEL or GroES or in both proteins, would not be able to efficiently refold misfolded proteins. Many antimicrobial agents including biocides are highly reactive chemicals and strongly interact with bacterial proteins (section 1.3.). For instance, aldehydes, alkylating and oxidizing agents all readily react with amino, carboxyl, sulphydryl and hydroxyl groups on proteins

causing irreversible modification of the protein structure (676). Protein coagulation can also occur as result of biocide action. Compounds such as chlorhexidine, phenols and QACs have all been reported to cause such effect (676). Protein damage caused by biocides in a GroEL-GroES deficient mutant would have a significant effect on the cell, as newly produced proteins may not be properly folded and misfolds in proteins may not be corrected. The latter would heighten the effect of the antimicrobial agent on the mutant, making it more sensitive to these compounds. Both triclosan and *ortho*-phthalaldehyde are known to act on proteins in the cell, which may explain the increased sensitivity of mutant N5-G6 to these agents.

A similar explanation may be given for the increase sensitivity of the mutant to the two aminoglycosides tested, amikacin and tobramycin, both known to act by inhibiting protein synthesis by binding to bacterial ribosomes. Inhibited protein synthesis coupled with inefficiency at folding proteins or correcting the misfolding in others could make the mutant become more sensitive to these antibiotics. It is interesting that the mutant showed increased resistance to meropenem, imipenem and piperacillin which are known to act by inhibiting bacterial cell wall synthesis. Site directed mutagenesis of each of the two genes (*groES* or *groEL*) individually and subsequent complementation analysis would give a better insight into the role of these genes in antimicrobial susceptibility.

3.5.2.2.3 Putative nucleoid-associated protein gene (*ndpA*)

Transposon insertion in mutant N3-B8 was located in a gene coding for a putative protein with high homology to a 37-kDa nucleoid-associated protein from *S. proteamaculans* and the NdpA from other *Enterobacteriaceae* including *Erwinia*, *Escherichia*, *Klebsiella, Salmonella* and *Yersinia.* Agar MICs demonstrated that the mutant had increased sensitivity to triclosan, but also increased resistance to cetylpyridinium chloride and chlorhexidine diacetate. The mutant also showed increased sensitivity to meropenem, imipenem, ciprofloxacin as well as azithromycin. Complementation of the *ndpA* gene, restored the wild type phenotype in the mutant, confirming that *ndpA* disruption was responsible for the phenotypic changes noted in the mutant.

The nucleoid-associated proteins, formerly known as histone-like proteins, constitute a superfamily of proteins that have genome structuring functions in bacteria. These proteins characterised by the relatively low molecular mass of their monomeric subunits and their ability to dimerize or oligomerize (643), are able to bind DNA and were referred to as histone-like proteins because their biochemical properties resemble eukaryotic histones (250, 822). Nucleoid-associated proteins are considered to be structural proteins setting the overall DNA conformation in the nucleoid, not only by wrapping or packaging DNA but also by introducing bending or coiling (250). The association of these proteins, however, influences not only the conformation but also other DNA processes such as replication, recombination, repair, and transcription (212, 244). There are many families of nucleoid-associated proteins isolated from different bacterial species. In *E. coli*, Azam and Ishihama (30) were able to express and purify 12 species of DNA-binding proteins. Five of these were found to bind to specific DNA sequences, while the other seven showed sequence-nonspecific DNA binding activity.

One of best described nucleoid-associated proteins are the histone-like nucleoid structuring protein (H-NS), best characterised in *E. coli* and other *Enterobacteriaceae.* This family of proteins not only contributes to bacterial chromosome architecture but can also generate nucleoprotein complexes in the vicinity of specific promoters to influence transcription (243). Studies have also shown that proteins belonging to the H-NS family can silence large AT-rich chromosomal segments that are probably acquired by horizontal gene transfer (627, 748). Evidence of effect of nucleoid-associated proteins on gene expression includes the actions of the Hha-YmoA family which has been shown to be involved in gene regulation in many Gram-negative bacteria. For example, *hha* and *ymoA* mutants exhibited varying phenotypes including altered plasmid supercoilling (141), increased insertion sequence transposition (701), and increased production of the toxin α-haemolysin in *E. coli* (354). Further studies showed that Hha controls the expression of other *E. coli* virulence factor genes (729, 730, 973), as well as the expression of many genes unrelated to pathogenesis (39). Balsalobre *et al.* (39) studied the effect of the *hha* mutation on the overall protein pattern of *E. coli* cells, and interestingly found that among the proteins whose expression was modified, both OmpA and protein IIAGlc of the phosphotransferase system could be identified. The latter enzyme participates in the regulation of the

synthesis of cyclic AMP (cAMP) and hence influences the catabolite repression system in the cell.

In *S. typhimurium*, Hha plays a role in the environmental regulation of the *hilA* gene, a transcriptional activator of invasion genes (276). Silphaduang *et al.* (983) found that Hha is the major repressor responsible for silencing virulence genes carried in *Salmonella* pathogenicity island 2 prior to bacteria sensing an intracellular environmental invasion. The *Y. enterocolitica* YmoA protein is a key regulator of virulence gene expression by environmental conditions. In addition to its role in the temperature-dependent production of the Yop proteins and YadA adhesin in *Y. enterocolitica* (193, 702), YmoA also modulates invasin production in *Y. enterocolitica* (268) and the type III secretion system in *Y. pestis* (486).

A number of mechanisms have been proposed by which H-NS effect gene expression: (i) binding to AT-rich sequences forming a nucleoprotein bridge which obstruct the access to RNA polymerase (842), (ii) interfering with either promoter clearance or RNA progression (642, 955, 975), and (iii) affecting DNA topology which changes transcription from supercoiling sensitive genes (718). Whatever way these proteins act, they clearly have an effect on gene expression and their influence extends over a wide range genes. Hence the expression of many genes in the NdpA-deficient mutant N3-B8, could have been effected. We can only speculate about the nature of these genes altered by the *ndpA* mutation. However, the increase in resistance to both cetylpyridinium chloride and chlorhexidine diacetate in the mutant suggests that the mutation either through lack of NdpA or due to a polar effect may have affected some kind of efflux system. Of particular interests is the gene encoding a putative bicyclomycin resistance protein (Bcr)/multidrug efflux system protein (EmrD), which is encoded downstream from *ndpA*.

Bicyclomycin has a weak antibiotic activity against certain Gram-negative organisms including *E. coli,* but is inactive against Gram-positive organisms (768). Bentley *et al.* (65) reported that the *bcr* gene from *E. coli* encoded a protein with homology to bacterial transmembrane proteins such as those mediating chloramphenicol and tetracycline resistance, and that it conferred resistance to a single drug, bicyclomycin. However, Nishino and Yamaguchi (770) found that the gene seems to confer a

moderate increase in resistance to some other compounds including, tetracycline, fosfomycin, kanamycin, and acriflavine.

As mentioned above the gene also showed high homology to EmrD, a proton-dependent secondary transporter from *E. coli* belonging to the MFS efflux superfamily. EmrD was first identified as an efflux pump for uncouplers of oxidative phosphorylation such as meta–chloro carbonylcyanide phenylhydrazone and tetrachlorosalicylanilide, which can rapidly arrest growth in bacteria by depleting the proton gradient (560, 747). In a later study, Nishino and Yamaguchi (770) showed that EmrD could also transport detergents such as benzalkonium and sodium dodecylsulfate. It is therefore possible that a polar effect from the mutation in *ndpA* gene somehow influenced the expression of the *bcr/emrD* efflux system downstream leading to the resistant phenotype seen in the mutant.

Efflux pumps are an important mechanism of bacterial intrinsic resistance to antimicrobial agents (section 1.5.1.2.). Evidence of efflux pumps presence in *S. marcescens* and their role in resistance to antimicrobial compounds has been accumulating in the last few years. Chen *et al.* (157) cloned genes responsible for drug resistance from clinical *S. marcescens* isolates which were highly resistant to antibiotics into the drug-hypersensitive strain of *E. coli,* KAM32. Clones carrying the *sedXY* genes were found to display reduce susceptibility to several antimicrobial agents including erythromycin, tetracycline, norfloxacin, benzalkonium chloride, ethidium bromide, acriflavine, and rhodamine 6G. Small increases in the MIC were also observed for ampicillin, triclosan and chlorhexidine gluconate (156). Similarity searches showed that SdeY is a member of the RND family of multidrug efflux proteins and SdeX is a member of the membrane fusion proteins. Investigators concluded that SdeXY was the first RND-type multidrug efflux pump to be characterised in *S. marcescens*, and that the pump had a wide range of antimicrobial agent substrates.

Berlanga *et al.* (69) suggested the presence of an efflux mechanism for ciprofloxacin and other fluoroquinolones in *S. marcescens*. This suggestion was investigated further by Kumar and Worobec (567), who studied the presence of such pump in a number of fluoroquinolones resistant *S. marcescens* clinical isolates. They confirmed the

presence of a proton-gradient-dependent efflux mechanism in *S. marcescens*. One clinical isolate, T-861 and a *S. marcescens* mutant UOC-67WL, both were shown to efflux ciprofloxacin and ofloxacin and to overexpress an AcrA-like protein. AcrA is part of the well characterised AcrAB pump in *E. coli* which belongs to the RND efflux family (1075). AcrAB multi-drug pump was shown to transport a number of antibiotics and microbial agents such as tetracycline, ciprofloxacin, chloramphenicol, fluoroquinolones, β-lactams, nalidixic acid, rifampin, and novobiocin as well as ethidium bromide, acriflavine, phenylethylalcohol, sodium dodecyl sulfate, and deoxycholate (636-638, 744, 780).

In fact Kumar and Worobec (567) revealed the presence of at least two AcrA-like proteins in *S. marcescens*. In addition, they sequenced a portion of the gene encoding the inner membrane component of an RND pump protein in the wild type *S. marcescens* strain UOC-67, and they reported that it showed similarity to the *mexF* gene expressed in *nfxC* mutants of *P. aeruginosa.* The *nfxC* mutants express the efflux pump, MexEF-OprN, and exhibit resistance to fluoroquinolones, imipenem, and chloramphenicol, and hypersusceptibility to β-lactam antibiotics (665). A follow up study on fluoroquinolones resistance in *S. marcescens* revealed the presence of two different loci involved in efflux in the organism. The first *sdeAB*, encoded a membrane fusion protein and an RND pump, and is responsible for the efflux of a diverse range of substrates including ciprofloxacin, norfloxacin, ofloxacin, chloramphenicol, sodium dodecyl sulphate, ethidium bromide, and *n*-hexane. The pump was overexpressed in multidrug resistant *S. marcescens* strains. The other loci, *sdeCDE*, coded for a membrane fusion protein and two different RND pumps but did not result in change in susceptibility to the above agents.

Kumar and Worobec (568) identified a *tolC*-like gene (*hasF*) in *S. marcescens* genome, with a protein product which had 80% amino acid homology with the *E. coli* TolC. The latter is part of the AcrAB-TolC multidrug efflux complex in *E. coli* responsible for transport of different varieties of antimicrobial agents (636-638, 744, 780). When the *hasF* was introduced to a *tolC*-deficient *E. coli*, the strain displayed 64-fold increase in resistance to sodium dodecyl sulphate and ethidium bromide, but no change was seen in susceptibility to fluoroquinolones or chloramphenicol. The

hasF product therefore was shown to play a role in the proton-gradient dependent efflux of at least sodium dodecyl sulphate and ethidium bromide.

Other efflux systems have also been reported in *S. marcescens* including the copper efflux pump CopA. The latter is a copper-translocating P-type ATPase which has been shown to be involved in copper resistance in *E. coli* (866). Williamson *et al.* (1148) demonstrated that inactivation of the *copA* gene homolog in the prodigiosin producing *S. marcescens* Sma 274, resulted in increased sensitivity to copper. This confirmed that CopA plays a role in the copper homeostasis in *S. marcescens* Sma 274. Only recently, Shahcheraghi *et al.* (971) characterised the first MFS efflux pump (SmfY) in *S. marcescens*. Introduction of the *smfY* gene from *S. marcescens* into the drug-hypersensitive *E. coli* KAM32 lead to increased resistance to a number of agents including norfloxacin, benzalkonium chloride, tetraphenylphosphonium chloride, methyl viologen, acriflavine, 4',6-diamino-2-phenylindol, and ethidium bromide. The pump is thought to play an important role in *S. marcescens* resistance to the common QAC antiseptic, benzalkonium chloride.

3.5.2.2.4 Putative cell envelope biogenesis operon genes

A number of biocide mutants had transposon insertions which were located in a putative cell envelope biogenesis operon consisting of at least four genes. These were *wbbD*, encoding a putative type 11 methyltransferase, *wbdA* and *wbdB* encoding for putative mannosyltransferase A, and B respectively (group 1 glycosyltransferases), and *wbpZ* encoding for another glycosyltransferase. Upstream of the putative operon was a *wzt*-like gene encoding a putative ATP binding component of an ATP-binding cassette (ABC) transporter involved in extracellular polysaccharide export. In *E. coli* serotypes O8 and O9a, Wzt is the nucleotide-binding component of the ATP-binding cassette involved in the polymannan O-antigenic polysaccharides synthesis via the transporter-dependent pathway (206). Downstream of the putative operon was a putative *gnd* gene coding for a putative 6-phosphogluconate dehydrogenase involved in carbohydrate transport and metabolism in the cell.

The biosynthesis of disaccharides, oligosaccharides and polysaccharides involves the action of hundreds of different glycosyltransferases. These enzymes catalyse the

transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. These bacterial enzymes are involved in various biosynthetic processes including exopolysaccharide biosynthesis, biosynthesis of the slime polysaccaride colanic acid and lipopolysaccharide core biosynthesis. In the latter context, many of the above genes were shown to be involved in this process. Lipopolysaccharides constitute a major component of the outer membrane of Gram-negative bacteria, and contribute greatly to the structural integrity and pathpogenecity of these organisms (272). They are composed of three parts: (i) lipid A, (ii) a core oligosaccharide, and (iii) an O-polysaccharide (O-antigen) made up of multiple copies of an oligosaccharide unit (600). Biosynthesis of the O-polysaccharide has been extensively studied and was shown to involve the action of many glycosyltransferases (538-540, 1183). Three major pathways have been proposed for O-polysaccharide biosynthesis: (i) *wzy*-dependent (62, 215, 380), (ii) ABC-transporter-dependent (671, 932), and (iii) synthase-dependent (849).

The O-antigenic polysaccharide portions of the lipopolysaccharide molecules in *E. coli* serotypes O8 and O9a (and its minor variant O9) are synthesised via an ABC transporter-dependent pathway (Figure 3.30). They involve the action of *wecA*, located outside the O-antigenic polysaccharide synthesis locus, and primes synthesis through the transfer of a *N*-acetylglucosamine-1-phosphate to the undecaprenol phosphate carrier lipid. O-antigenic polysaccharide polymerization is achieved via the action of mannosyltransferases products of the *wbdA, wbdB*, and *wbdC* genes, located in the O-antigenic polysaccharide biosynthesis cluster (540). The WbdD enzymes of the *E. coli* O8 and O9a biosynthesis systems are required for the addition of the non-reducing terminal modifications to the nascent polymer (175). The addition of these terminal residues is required not only for polymerization termination and chain-length regulation but also for the export of O-antigenic polysaccharides from the cytoplasm. Wzm and Wzt constitute the ABC transporter required for the export system (540). The number of O-units per lipopolysaccharide molecule is not constant, but usually the majority of molecules have chain lengths clustered around a modal value. This characteristic O-antigen modal value is determined by the long chain determinant Wzz (previously Cld or Rol) (52, 53).

Because they are vital components of lipopolysaccharides molecule, which in turn are major components of the outer membrane of Gram-negative bacteria, relatively minor changes in the composition of the O-antigenic polysaccharide chains brought up by defective genes would have substantial implications on the cell membrane and its biological characteristics. Mutations in gene coding for glycosyltransferases have been shown to cause such effects. Kido and Kobayashi (538) found using site-directed mutagenesis of the *wbdA* gene, that a single amino acid substitution in the WbdA protein of *E. coli* O9, converted the O9 polysaccharides into O9a. Riley *et al.* (881) found that mutation in the *wbbD* gene of the O7 lipopolysaccharide biosynthesis cluster in *E. coli* VW187, coding for a glycosyltransferase, lead to the mutant not being able to form O7 lipopolysaccharides. Yi *et al.* (1183) functionally inactivated the *wbnI* gene coding for a glycosyltransferase involved in the O-polysaccharide biosynthesis in *E. coli* O86. They reported that the mutant strain produced a different polysaccharide lacking a side chain residue at a lower yield compared with the wild type.

Yethon *et al.* (1182) demonstrated that mutation of the core glucosyltransferase (a glycosyltransferases which enable the transfer of glucose) encoded by *waaG* of *E. coli*, resulted in alterations in the lipopolysaccharide molecule. The mutation also destabilized the outer membrane and compromised its barrier function. The mutant showed increased susceptibility to sodium dodecyl sulfate and novobiocin (1182). Grebe *et al.* (361) reported that piperacillin resistance in *S. pneumoniae* was mediated by mutations in a novel gene, *cpoA*, that also confer transformation deficiency and a decrease in penicillin-binding protein 1a. CpoA was shown to be a homologue to glycosyltransferases that act on membrane-associated substrates, such as enzymes functioning in lipopolysaccharide core biosynthesis of Gram-negative bacteria. Investigatores concluded that CpoA, a putative glycosyltransferase, was a novel resistance mechanism against β-lactams (361).

In this study, transposon insertions in 4 mutants (N4-F6, N2-A8, N2-F1 and N5-G1) were located within the putative *wbdA* gene of *S. marcescens* Db11. On agar, all these mutants showed increase susceptibility to chlorhexidine diacetate and three out of the four (N4-F6, N2-A8, and N5-G1) also showed increase sensitivity to cetylpyridinium chloride. All four *wbdA*-defective mutants showed increased sensitivity to the two

aminoglycosides tested, amikacin and tobramycin, and three of them showed increased resistance to meropenem. A fifth mutant (N5-D9) had a transposon insertion in the putative *wbpZ* gene and showed increased sensitivity to triclosan on agar. Evidence of increase sensitivity to the killing effect of chlorhexidine diacetate and cetylpyridinium chloride was also observed. Similar to the above four *wbdA* mutants, the *wbpZ*-deficient mutant also showed increased sensitivity to two amikacin and tobramycin, and increased resistance to meropenem. It was also more resistant than the wild type to ciprofloxacin, piperacillin, and ceftazidime.

Given the important role glycosyltransferases play in the determining the structure of the outer membrane of the cell, and their effect on its function as a permeability barrier, it is not surprising that mutants defective in such proteins would have altered antimicrobial susceptibility. It can be speculated that transposon disruption of the *wbdA* or *wbpZ* genes could have led to changes in lipopopysaccharides of the cell, leading to alteration in the outer membrane structure and properties. This in turn would have caused changes in the membranes permeability making these mutants more sensitive to the actions of biocides. The two main phenotypic changes noted in the mutants were increase sensitivity to chlorhexidine and cetylpyridium chloride. Both of these biocides are membrane-active agents known to target the outer membrane of bacteria by binding to phosphate head groups and fatty acid chains in phospholipids, affecting the membrane potential and electron transport chains, and causing membrane damage.

Indeed evidence of increased susceptibility to chlorhexidine due to mutation in a glycosyltransferase has already been presented by Rees (860). In a study on the molecular basis of triclosan and chlorhexidine resistance in *B. cenocepacia*, Rees (860) isolated a chlorhexidine mutant, also showing resistance to the QAC cetrimide, which had a transposon mutation within the *gltA* gene coding for a glycosyltransferase. Inactivation of the gene affected other cellular processes in the mutant including, reduced growth rate and increased lag phase, reduced viability following long term storage (-80^{0}C), reduced motility, and a detectable change in its lipopolysaccharide profile when compared to the wild type.

Changes in membrane permeability may have facilitated the passage of aminoglycosides into the cell, leading to the increase sensitivity to amikacin and tobramycin seen in all the mutants. A large number of the mutants showed increased resistance to meropenem, a carbapenem belonging to the β-lactam antibiotics. Members of this family act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins. Grebe *et al.* (361) showed that resistance to β-lactams can arise from mutation in a putative glycosyltransferase which also lead to decrease in a penicillin-binding proteins 1a in *S. pneumoniae*. It might be possible that a similar effect may have occurred due to mutation in the *wbdA* gene which lead to increased resistance to meropenem. Similar explanation could be given for the increased resistance of the *wbpZ* mutant to the β-lactams meropenem, piperacillin, and ceftazidime.

The position of the transposon insertion in the *wbpZ* within the putative lipopolysaccharide biogenesis operon may explain its reported phenotype. Although the mutation was within the same putative operon which included *wbdA*, the *wbpZ* mutant did not show increased sensitivity to either chlorhexidine or cetylpyridium chloride, as did the *wbdA* mutants. This could be explained by its position within the putative operon. The *wbpZ* gene is located downstream from *wbdA* toward the end of the operon (Figure 3.15). This meant unlike the case for a *wbdA* mutation, mutation in the *wbpZ* gene would not stop the synthesis of most of the operon-encoded proteins and other proteins needed for lipopolysaccharide synthesis (Wzt, WbbD, WbdA, and WbdB). A mutation in the *wbpZ* gene would therefore have less effect on the lipopolysaccharide synthesis pathway than a mutation in the *wbdA* gene. Further investigations including complementation of both the *wbdA* and the *wbpZ* mutants are needed to explore the above speculations.

3.5.2.2.5. Putative outer membrane biogenesis operon gene (*wzzE*)

Two mutants 19-D3 and 10-B6 with predominantly increased biocide resistance phenotype had transposon insertion mapped into a putative *wzzE* gene. As mention earlier (section 3.5.2.2.4), Wzz proteins regulate the degree of polymerization of the O-antigen subunits in lipopolysaccharide biosynthesis. The disrupted gene appeared

to be part of a putative cell lipopolysaccharide biosynthesis operon composed of at least three other genes (Figure 3.17). These were *wecB* (*rffE*) and *wecC* (*rffD*), involved in the synthesis of enterobacterial common antigen, a mannosaminuronic-acid-containing exopolysaccharide that can be attached to core lipid A in *Enterobacteriaceae* (692), and *rfe*, also involved in biosynthesis of the enterobacterial common antigen, as well as certain O-polysaccharides (550, 877). The biological function of the Wzz proteins is well known from studies on *wzz* mutants. These typically display a random, non modal distribution of O-antigen chain lengths compared with the wild type (52, 53, 306). Franco *et al.* (307) reported that O-antigen chain length heterogeneity in *E. coli* strains is a result of amino acid sequence variation of the Wzz protein, after they showed that the model value of chain length in *E. coli* O1, O2, O7 and O157 could be changed by specific amino acid substitutions in *wzz*.

Wzz proteins belong to the "Polysaccharide Co-Polymerases" super family, members of which are involved in the chain length regulation of polysaccharide including O-antigen, capsule polysaccharides, and exopolysaccharides (56). Wzz proteins are characterised by two transmembrane segments located in the amino-terminal and carboxy-terminal, with a large hydrophilic loop located in the periplasm (724). Wzz genes have been identified in many Gram-negative species, suggesting an importance in survival and pathogenesis. Burrows *et al.* (129) characterised *wzz* gene in *P. aeruginosa* and showed that *wzz* knockout mutants expressed O-antigens with chain lengths markedly different from their parental strains. Murray *et al.* (739) reported that *S. typhimurium* posses two functional *wzz* genes which results in bimodal O-antigen chain distribution. One gene (wzz_{ST}) is responsible for long modal length lipopolysaccharides with 16-35 O-antigen repeat units, and wzz_{fepE} is responsible for very long modal length containing over 100 O-antigen repeat units.

O-polysaccharide moieties of lipopolysaccharides have been shown to mediate many biological effects, including resistance to killing by normal, non-specific serum (740, 837), resistance to phagocytosis by monocytes, and resistance to killing by cationic peptides (854, 1135). O-antigens are important in virulence and their biological activities have been correlated to their size and distribution on the surface of the bacterial cell (379, 494, 497, 641, 819). Given the position of the *wzzE* gene in the *S.*

marcescens Db11 genome, disruption of the gene could have altered the expression of not only WzzE, but also that of WecB and WecC. This would result in alteration of the O-antigen chain length and changes in lipopolysaccharide structure, therefore outer membrane permeability.

The WzzE-deficient mutant 19-D3 had increased resistance to cetylpyridinium chloride, chlorhexidine diacetate and *ortho*-phthalaldehyde. The other WzzE-deficient mutant 10-B6 was similar to 19-D3 in that it showed increased resistance to *ortho*-phthalaldehyde, but interestingly was sensitive to cetylpyridinium chloride. Antibiotic susceptibility profiles for the two mutants showed that both mutants exhibited increased resistance to 3 out of the 10 antibiotic tested, and increased sensitivity to one antibiotic. However, there were no similarities between the antibiotic susceptibility profiles of the two mutants. 19-D3 showed increased resistance to the two aminoglycosides amikacin and tobramycin as well as to sulphonamide trimethoprim/sulfamethoxazole, and was more susceptible than the wild type to the β-lactam meropenem. Mutant 10-B6, showed increased resistance to β-lactams (piperacillin, ceftazidime and imipenem) but was more sensitive than the wild type to the quinolone, ciprofloxacin. The general phenotypic trend in both mutants was increased resistance to biocides and antibiotics and it can be explained by alteration in the outer membrane barrier as result of deactivation of either the *wzz* gene alone or *wzz* and the genes downstream from it in the putative operon.

However, the two mutants disrupted in the same gene showed markedly different phenotypic changes especially in relation to their antibiotic susceptibility. This could also be explained by referring to the above study by Franco *et al.* (307), who reported that single amino acid changes in the Wzz protein, resulted in expression of different O-antigen chain lengths in *E. coli*. When mapping the location of the transposon insertions in mutants 19-D3 and 10-B6, it was observed that, although within the same gene, insertions were not in the exactly same location in the two mutants. In the unlikely, but possible, event that an altered WzzE protein was still expressed in one or both mutants, the O-antigens of the mutants would be different. This could result in differences in antimicrobial susceptibility. A similar situation was reporter in the above study by Burrows *et al.* (129), where *P. aeruginosa* knock out mutants, using gentamicin resistance cassette, still expressed O-antigens with chain lengths markedly

different from their parental strains. This is only a speculation, and further tests are needed to confirm or reject this hypothesis. RT-PCR could be used to check at the mRNA level whether the disrupted *wzzE* gene is transcribed in either mutant. Similarly, translation and expression of the WzzE in the mutants could be checked by determining their protein profiles.

3.5.2.2.6 Putative succinate dehydrogenase hydrophobic membrane anchor protein gene (*sdhD*)

Transposon insertion in mutant 22-D5 was shown to be in a putative succinate dehydrogenase hydrophobic membrane anchor protein gene (*sdhD*). The latter appeared to be part of a putative succinate dehydrogenase operon involved in energy production and conversion in the cell, which includes at least three genes (*sdhA*, *sdhB*, and *sdhD*). On agar, the mutant demonstrated increase susceptibility to *ortho*-phthalaldehyde and cetylpyridinium chloride. The mutant also showed reduce growth rate and increased sensitivity to both meropenem and tobramycin.

Succinate dehydrogenase, also referred to as succinate:quinine oxidoreductase, or Complex II, is a functional member of both the citric acid cycle and aerobic respiration (944). Succinate dehydrogenase catalyses the oxidation of succinate to fumarate coupled with the reduction of ubiquinone in the membrane (145). The action of succinate dehydrogenase is shared by fumarate reductase, which is involved in a form of anaerobic respiration with fumarate as the terminal electron acceptor (558). Collectively, succinate dehydrogenase and fumarate reductase are referred to as succinate:quinine oxidoreductases and are predicted to share similar structure (579). The complexes consist of two hydrophilic, and one or two hydrophobic, membrane-integrated subunits (391). The larger hydrophilic subunit A is a flavoprotein, and the smaller subunit B is an iron-sulfur protein. In *E. coli*, these are SdhA and SdhB respectively. The succinate dehydrogenase in *E. coli* has two hydrophobic membrane-integrated subunits (ShdC and ShdD) which contain one heme *b* and provide the biding site for ubiquinone (145).

Given the important role succinate dehydrogenase plays in energy production and electron transfer in the cell, it is not surprising that the SdhD-deficient mutant showed

decreased growth rate compared to the wild type. Given the position of *sdhD* in *S. marcesence* Db11 genome at the beginning of the putative succinate dehydrogenase operon (Figure 3.20), mutation in this gene has the potential to not only disrupt *sdhD* but also alter the expression of the two hydrophilic subunits SdhA and SdhB. This could result in a complete disruption of the electron transfer chain in the cell. Indeed such disruption has been reported by Weiner *et al.* (1133) who identified a mutation within the membrane-intrinsic domain of the terminal electron-transfer enzyme fumarate reductase of *E. coli*. As mentioned above fumarate reductase and succinate dehydrogenase have the same catalytic function and have similar structures. The fumarate reductase of *E. coli* is a complex iron-sulfur flavoenzyme composed of four subunits, FrdA and FrdB (a membrane-extrinsic catalytic domain) and FrdC and FrdD (a transmembrane anchor domain). Weiner *et al.* (1133) found that a mutation within the transmembrane anchor domain (FrdC) altered the electron transfer properties of the iron-sulfur and flavin-redox centers of the catalytic domain. This resulted in impairment of the functional electron flow in the electron transport chain. These results confirmed the important role of the anchor subunit in functional electron transport, and alterations within this subunit could have major effects on the electron transport in the cell.

In addition, a number of biocides are known to exert their action by altering or targeting the membrane potential and electron transport chains within bacteria. Biocides acting on the cytoplasmic membrane level may inhibit the energy processes in the cell by disrupting the proton motive force (section 1.3). The latter is involved in active transport, oxidative phosphorylation and ATP synthesis in bacteria (461, 714, 715), and is generated by oxidation-reduction reactions occurring during electron transport. In a mutant where the electron transport chain is impaired, such biocides could have a more substantial effect. Biocides known to have such mode of action are the surface-active agents chlorhexidine and QACs including cetylpyridium chloride. For instance, studies on *E. coli* have shown that chlorhexidine collapses the membrane potential of the bacterium (574). Barrett-Bee *et al.* (48) studied the membrane destabilising action of chlorhexidine in a number of bacterial species including *E. coli*, *S. aureus*, *Morganella morganii* and *P. aeruginosa*. They reported that the agent cause inhibition of oxygen utilisation in the bacteria that was related to fall in cellular ATP levels. Therefore it is not surprising that the SdhD-deficient

mutant was more sensitive to the membrane-active agent cetylpyridium chloride than the wild type.

3.5.2.2.7 Putative pili operon gene

Agar MICs showed that mutant N5-B5 was more sensitive to triclosan and *ortho*-phthalaldehyde than the wild type but had increased resistance towards cetylpyridinium chloride. The mutant exhibited increased sensitivity to the two carbapenems, meropenem and imipenem, as well as to piperacillin. Mutant N5-B5 had a transposon insertion located within a putative FimC encoding gene. The putative protein showed high homology to chaperone PapD of *E. coli*. The disrupted gene was flanked to the left by a gene coding for a putative FimA protein, a member of the adhesin superfamily, and to the right by a gene coding for a putative fimbrial biogenesis outer membrane usher, with homology to the PacC of *E coli*.

An important initial event during the establishment of infections by pathogenic Gram-negative bacteria is the attachment of bacteria to host cell receptors. This process is mediated by adhesive surface organelles termed pili or fimbriae (466, 998). Gram-negative bacteria produce a diverse array of pili that mediate this microbe–microbe and host–pathogen interactions important in the development of disease. Pili are large, heterooligomeric protein filaments anchored to the bacterial outer membrane, and their biogenesis requires the orchestration of a complex process that includes protein synthesis, folding, secretion, and assembly (467). Periplasmic chaperones are part of a general secretory pathway required for the assembly of pilli, and act by stabilizing pilus subunits in the periplasm through the formation of distinct periplasmic complexes (468, 564). The PapD-like proteins are the best characterised periplasmic chaperones, and currently there are more than 30 known such chaperones facilitating the assembly of both pilus and non-pilus organelles (469). Much of the knowledge about the function of PapD-like periplasmic chaperones comes from the study of PapD and FimC, which assemble P and Type 1 pili, respectively, in *E. coli* (465, 499, 610).

Type 1 pili are produced by nearly all *Enterobacteriaceae* (104), they are 0.5-2 μm long and 7 nm wide filaments (548) consisting of a short tip fibrillum containing the

FimH adhesin, joined to the distal end of a pilus rod. Overall, nine different *E. coli* proteins are involved in the biogenesis of Type 1 pili, whose genes are clustered at the *fim* operon (467). FimA is the main structural pilus subunit and comprises about 98% of all pilus proteins, while the residual 2% are comprised by FimF, FimG, the adhesin FimH, and possibly FimI (548). The outer membrane protein FimD anchors the pilus to the bacterial surface and represents its assembly platform, while the cytosolic proteins FimB and FimE regulate the transcription of pilus genes (547). The assembly of the pilus relies on FimC, a pilus chaperone in the periplasm (499). The chaperone functions in concert with an outer membrane usher protein, FimD. This oligomeric protein allows translocation of the pilus subunits to the cell surface and their incorporation into the growing pilus (238, 466, 548).

Given the location of the transposon insertion in mutant N5-B5 in the middle of the putative pilus biosynthesis operon, it is possible that the mutation would have disrupted not only the expression of FimC but also other proteins transcribed downstream such as the usher FimD. The result would be a significant disruption of the whole pilus biosynthesis apparatus in mutant N5-B5. Given the important role of pili in determining the outer surface of cells, and their vital contribution to virulence, it is not surprising that mutants with altered pili biosynthesis would exhibit different antimicrobial susceptibility profiles. Chen *et al.* (155) reported that different mutations in the *pilQ* gene encoding a member of the secretin family of proteins which functions in type IV pilus organelle biogenesis in *Neisseria gonorrhoeae* (247), lead to mutants exhibiting varying phenotypes. These included decreased piliation and transformation efficiency, loss of hemoglobin utilization phenotype, decreased entry of free heme, increase sensitive to the toxic effect of free heme, hypersensitivity to the detergent Triton X-100 and multiple antibiotics. These results show that disruption in pilus biosynthesis could result in alteration in membrane permeability. It is interesting that mutant N5-B5 showed increased sensitivity to β-lactams known to act on cell wall synthesis.

3.5.2.2.8 Putative outer membrane protein A gene (*ompA*)

Transposon insertion in mutant 12-F6 was located within an ORF that coded for a 371 amino acid long putative protein with high homology to the outer membrane protein

A (OmpA) from a number of *Enterobacteriaceae*, including species of *Serratia*, *Yersinia*, *Shigella* and *Klebsiella*. On agar, mutant 12-F6 showed increased susceptibility to triclosan, *ortho*-phthalaldehyde, and chlorhexidine diacetate. It also exhibited increased sensitivity to chloramphenicol, azithromycin, and piperacillin. It was however more resistant than the wild type to the two aminoglycosides, amikacin and tobramycin, and to imipenem.

OmpA is one of the best characterised major outer membrane proteins in *E. coli*. In strain K-12, the protein is 35-kDa and is one of the few abundant polypeptides in its outer surface spanning the membrane (159). The *E. coli* K-12 gene, *ompA*, has been cloned, and its nucleotide sequence has been determined (55, 428, 731, 732). Extensive evolutionary and functional studies followed, which revealed that in *E. coli* the protein has a two domain configuration in which the amino-terminal with 177 amino acid residues is in the membrane, while the carboxyl-terminal consisting of 148 residues is situated in the periplasm (100, 963). OmpA has been identified in all Gram-negative bacteria tested which include 17 genera (58), among these are *Serratia*, *Shigella*, *Yersinia*, *Klebsiella, Salmonella*, and *Enterobacter*. Evolutionary studies found that although OmpA was highly conserved among *Enterobacteriaceae* (551), it was suggested that during the evolution of this family, some parts of the OmpA polypeptide, referred to as "variable regions", had undergone extensive divergence, and are probably all located on the extracellular side of the outer membrane. The other parts which had remained unchanged, called "constant regions", are located within the membrane or periplasm (95-97, 316).

In combination with lipoproteins (98), OmpA is thought to contribution to the structural integrity of the outer membrane and the generation of normal cell shape (996). In addition to its structural role, OmpA serves as a receptor of colicin and several phages (301, 723) including K3 (399) and Ox2 (528), and it is required in F-conjugation (553, 962). OmpA has been shown to have porin activity producing a diffusion channel allowing a slow penetration of small solutes (1027). The protein has also been implicated in various host defense processes, although this role seems to be indecisive. OmpA has been shown to be of importance for the invasive capacity of *E. coli*, and for resistance to antibacterial activity of serum, which suggest that it acts as a virulence factor. Indeed OmpA mutants were reported to be significantly less

virulent in embryonic chicken and neonatal rate models (1134), and their ability to invade brain microvascular endothelial cells to be largely reduced (838). On the other hand, OmpA is a direct target for neutrophil elastase, and OmpA deficient mutants were shown to resist degradation by the latter and to survive better than the wild type *E. coli* inside neutrophils (60). Fu *et al.* (318) reported that OmpA deficient *E. coli* activated human neutrophils and were more susceptible than the wild type to membrane-acting bactericidal peptides. Therefore OmpA can facilitate the immune clearance of the bacteria from the host. An *E. coli* deletion mutant was also shown to be significantly more sensitive than the parent strain to sodium dodecyl sulfate, cholate, acidic environment, high osmolarity, and pooled human serum (1125).

Other outer membrane proteins have been identified in *S. marcescens*, many of which were described as porins. Malouin *et al.* (651) reported the presence of a suspected 41-kDa porin in the outer membrane of *S. marcescens* as well as the OmpA. They also concluded that *S. marcescens* possesses a greater permeability barrier than *E. coli* because of the expression of this pore-forming protein. The pore size of the protein was calculated at 1.06 nm, and had conductance intermediate between those of *E. coli* K-12 OmpC and OmpF porins. A later study by Puig *et al.* (843) reported that the 41-kDa porin band reported by Malouin *et al.* (651) was actually a combination of three separate outer membrane proteins. They determined that *S. marcescens* has at least four major outer membrane proteins, named Omp1 (42-kDa), Omp2 (40-kDa), Omp3 (39-kDa), and OmpA (37-kDa). They also reported similarity in behaviour between Omp2 and Omp3 from *Serratia* with OmpC and OmpF of *E. coli*, suggesting that they are both porins. Omp1 however, did not correspond to any protein in *E. coli* outer membrane. In a follow up study, Ruiz *et al.* (892) determined the molecular and functional characteristics of Omp1. The protein was cloned and sequences and showed homology with the family of outer membrane porins that comprises the general porins of enteric bacteria. Omp1 was shown to be highly cation-selective and that it has a single channel conductance similar to that of other porins from enteric bacteria. Expression of the Omp1 in a porin deficient *E. coli* strain conferred high susceptibility to different hydrophilic antimicrobial agents such as β-lactams and chloramphenicol. On the other hand no changes in susceptibility were observed for ciprofloxacin and tetracycline.

The literature is full of reports linking expression of outer membrane proteins, including OmpA, to changes in antimicrobial susceptibility. Porins, been shown to not only be involved in the passage of molecules through the cell but also to alter the cell surface hydrophobicity of *S. marcescens*, which affects its permiability. Mallick (649) studied the cell surface hydrophobicity in pigmented and non-pigmented *S. marcescens* mutants, and reported the presence of an extra 40-kDa outer membrane protein in the highly hydrophobic non-pigmented mutant, concluding that this protein may be responsible for higher surface hydrophobicity of the mutant. The involvement of outer membrane proteins in determining the cell surface hydrophobicity of *S. marcescens* was also reported by Bar-Ness and Rosenberg (42) who demonstrated that a 70-kDa outer-surface protein was promoting cell-surface hydrophobicity of *S. marcescens* RZ known to posses pronounced outer membrane hydrophobicity.

Clark (173) studied imipenem resistance in two *Acinetobacter baumannii* isolates (A-1 and A-24), and reported that this resistance was related to decreased expression of a 33-36-kDa outer membrane protein. In another study, Limansky *et al.* (609) reported similar results when they investigated imipenem resistance in multi-drug resistant isolates of *A. baumannii*. They found that resistance corresponded with the loss of a 29-kDa polypeptide from the outer membrane of the organism. No carbapenemase activity was detected in any of the strains isolated.

Imipenem resistance has also been linked to loss of outer membrane proteins in a number of other bacterial species. Bradford *et al.* (91) described that imipenem resistance in *K. pneumoniae* can occur when high levels of β-lactamases are produced in combination with loss of a major (42-kDa) outer membrane protein. Chow and Shlaes (163) noted that an imipenem-resistant strain of *E. aerogenes* showed loss of a 40-kDa outer membrane protein, decreased expression of 42- and 44-kDa proteins, and increased expression of a 50-kDa protein in its outer surface, when compared with imipenem-susceptible clinical isolates. They concluded that the 40-kDa protein might be required for the normal diffusion of the antibiotic across the outer membrane of *E. aerogenes*. Buscher *et al.* (130) reported that imipenem resistance in *P. aeruginosa* was linked to marked decrease oh either a 46- or 45-kDa protein in the outer membrane. In a latter study Martinez-Martinez *et al.* (663) demonstrated that decreased activity of imipenem in a *P. aeruginosa* strain eluted from siliconized latex

urinary catheters, was related to loss of an OprD-like protein and to the expression of a new 50-kDa outer membrane protein.

Outer membrane proteins have been linked to resistance to other antimicrobial agents in *P. aeruginosa*. Gimeno *et al.* (351) studied the outer membrane protein profiles of 122 *P. aeruginosa* isolates and related the results to imipenem, ceftazidime, and ciprofloxacin resistance. They noted alterations in the expression of porins OprC, OprF, and OprD in some of the isolates. Nicas and Hancock (756) reported that increased resistance to the chelator ethylenediaminetetraacetate and to the cationic antibiotic polymyxin B in *P. aeruginosa* cells grown in magnesium-deficient medium, was related to increased level of outer membrane protein H1. They proposed that the protein acts by replacing the magnesium at a site on the lipopolysaccharide which can otherwise be attacked by the cationic antibiotic or ethylenediaminetetraacetate. In a study by Li *et al.* (604), it was found that OprF, the most abundant outer membrane protein in *P. aeruginosa* PAO1161, was absent in a toluene-resistant strain. Investigators proposed that OprF could be an important protein in the diffusion of toluene across the membrane of *P. aeruginosa.*

Association between outer membrane proteins OmpW and STM3031 and ceftriaxone resistance in *S. typhimurium* has been reported (454). Similarly, carbapenem resistance in *E. coli* was linked to deficiency in a 38-kDa outer membrane protein (1006), and ceftazidime susceptibility to expression of 37- and 39-kDa outer membrane proteins in the same organism (35). In *S. dysenteriae*, β-lactam permeability was lower across a clinical isolate which lacked a 43-kDa outer membrane protein compared with a β-lactam sensitive strain expressing it (519). Cefamandole resistance in *K. pneumoniae* was linked to lack of the outer membrane proteins, OmpF (1087).

Other authors correlated reduced amounts of outer membrane porins with increase resistance to β-lactams in *S. marcescens*, and showed that highly resistant strains may be defective in outer membrane proteins used for β-lactams penetration (314, 315). Gutmann *et al.* (388) studied *in vitro* mutants of *Klebsiella*, *Enterobacter*, and *Serratia* cross-resistant to nalidixic acid, trimethoprim, and chloramphenicol that were similar to mutants found *in vivo*. They found that the sole mechanism for this type of

resistance appeared to be a reduction in permeability of the cell envelope. They also noted that in the outer membrane of the mutants, the amount of at least one major protein, possibly a porin, with a molecular size of approximately 40-kDa, was decreased. They concluded that the resistance seemed likely to be due to the reduction in quantity of outer membrane proteins, possibly porins. Traub and Bauer (1071) reported that *S. marcescens* clinical isolates which had altered outer membrane proteins were significantly less susceptible to amikacin, cefotaxime and lamoxactam than the wild type strains which were susceptible to these agents.

Hashizume *et al.* (404) studied the porin expression of two clinical isolates of *S. marcescens* which over produced cephalosporinase and had decreased outer membrane permeability in comparison with reference strains. Investigators isolated three outer membrane proteins, an OmpF-like (45-kDa) porin, and two OmpC-like (44- and 43-kDa) porins. They noted that the clinical isolates which overexpressed the 44-kDa or 43-kDa porins but had no significant changes in expression of the other outer membrane proteins, had significantly lower outer membrane permeability compared with a reference strain. After characterising the 40-kDa OmpC (472) and the 41-kDa OmpF (473) porins from *S. marcescens*, Hutsul *et al.* (473) concluded that these general diffusion porins produced by *S. marcescens* are similar to *E. coli* OmpF and OmpC.

As OmpC and OmpF from *E. coli* and other enterobacteria serve as β-lactam penetration channels, the role of the *S. marcescens* OmpF and OmpC in resistance to these agents was investigated by Weindorf *et al.* (1132). They looked at the contribution of both porins in high and low level resistance to β-lactam antibiotics in *S. marcescens* clinical isolates. They reported that low level resistance was caused by overproduction of β-lactamase alone, while high level resistance was due to β-lactamase overproduction as well as defects in OmpF or OmpF and OmpC. The results mirrored those reported by Hechler *et al.* (414), and demonstrated that the *S. marcescens* porins contribute to the high level resistance to β-lactams. Hechler *et al.* (414) studied antibiotic resistance in *S. marcescens* clinical isolates, and looked at the relative contributions to resistance by the functioning of periplasmic β-lactamase, synthesis of this enzyme, and limitation of antibiotic penetration by the bacterial outer membrane. They reported that although low level resistance to cefotaxime,

ceftizoxime, ceftazidime, aztreonam and latamoxef was due to solely the overproduced β-lactamase, high resistance was due to not only β-lactamase action but also to decreased permeability to antibiotics.

The role of *S. marcescens* outer membrane in the accumulation of quinolones has been investigated by Berlanga *et al.* (69). They studied the passage of quinolones through the outer membrane in both lipopolysaccharide-deficient and porin deficient mutants. They reported that the lipopolysaccharide layer formed a very efficient barrier for highly hydrophobic quinolones such as nalidixic acid. On the other hand, quinolones with lower hydrophobicity were shown to pass preferentially though the water filled Omp3 porin channels.

Guasch *et al.* (381) reported a 17-kDa outer membrane protein Omp4 closely resembling a family of small outer-membrane proteins of *Enterobacteriaceae* whose known functions appear to be related with virulence. They reported that overexpression of this protein decreased the amount of OmpA, OmpF and/or OmpC in the cell. Omp4 was reported to confer partial resistance to bacteriocin 28b when expressed in *E. coli.* (381). Evidence from the characterised *S. marcescens* outer membrane proteins suggest that these proteins and possible other uncharacterised membrane proteins play a role in the antimicrobial resistance of the organism.

Given the abundance of OmpA in Gram-negative bacteria, and its structural and biological functions, changes in susceptibility seen in the *S. marcescens* Db11 OmpA mutant are not surprising. Loss of OmpA from the mutant might have caused structural changes in the outer membrane leading to alteration in its permeability barrier. Increase susceptibility to triclosan, *ortho*-phthalaldehyde, and chlorhexidine diacetate suggest that OmpA might not be involved in the mechanisms by which these agents enter the *S. marcescens* Db11 cell. Moreover, the fact that the mutant did not show changes in sensitivity to cetylpyridinium chloride, suggests that the two membrane active agents (chlorhexidine and cetylpyridinium chloride) may cross the outer membrane via different mechanisms.

The antibiotic susceptibility profile of the OmpA mutant, showed that it had increased susceptibility to chloramphenicol, azithromycin, and piperacillin, but was more

resistant than the wild type to two aminoglycosides, amikacin and tobramycin, and to imipenem. Referring to the above reports of altered antibiotic susceptibility linked to outer membrane protein changes, it can be seen that the antibiotic phenotype of the OmpA mutant could be explained. Imipenem resistance has been linked to loss of outer membrane proteins in many bacterial species (see above), and it was the case in this study. OmpA might therefore be one of the mechanisms by which imipenem gain access to the *S. marcescens* Db11 cell. Moreover, *E. coli* OmpA mutants have been shown to resist the bactericidal effects of neutrophil elastase, a serine protease that hydrolyzes many proteins. It is therefore interesting that *S. marcescens* Db11 OmpA mutant became more resistant to the two aminoglycosides amikacin and tobramycin; both know to act at the protein level.

The OmpA from *S. marcescens* has been cloned and sequenced (95), and it was shown that the three regions of the protein likely to be exposed on the cell surface were different from the corresponding regions of *E. coli* polypeptides and all other OmpA proteins tested. Information about the nucleotide sequence of the OmpA gene in *S. marcescens* was used to complement the OmpA mutant. To make sure OmpA would be expressed in the complemented mutant, the DNA fragment cloned into the mutant contained the whole OmpA gene along with its promoter region and translation start and stop signals reported by Braun and Cole (95) (Figure 3.23). The complemented mutant however did not show restoration of the wild type phenotype. Further analysis including RT-PCR and comparison of the mutant's protein profile with that of the wild type showed that OmpA was transcribed in one mutant construct but not translated. Therefore, expression of the OmpA was stopped somehow at the translation step. An explanation for this is difficult to derive.

3.5.2.2.9 Putative 6-phosphofructokinase gene (*pfkA*)

Transposon insertion in the genome of mutant N6-B2 was located within a putative phosphofructokinase encoding gene (*pfkA*). Phosphofructokinase is a key enzyme in glycolysis and catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-biphosphate. Glycolysis is the initial step of most carbohydrate catabolism, and is responsible for converting glucose to pyruvate and generating ATP in the process. The main functions of glycolysis are:

- Generation of high-energy molecules (ATP and NADH)
- Production of pyruvate
- Production of variety of six- and three-carbon intermediates compounds, which may be used at various steps in the process and for other cellular purposes.

Glycolysis is important for maintaining a supply of carbon skeletons for biosynthesis, as many of the metabolites in the glycolytic pathway are used by anabolic pathways. These pathways include

- Gluconeogenesis, which generates glucose from non-sugar carbon substrates such as pyruvate, lactate, glycerol, and glucogenic amino acids.
- Lipid metabolism
- The pentose phosphate pathway, which is a cytosolic process that serves to generate NADPH and the synthesis of pentose (5-carbon) sugars.
- The citric acid cycle, which in turn leads to both amino acid and nucleotides biosynthesis

Given the central role of glycolysis in catabolism, maintaining energy levels in the cell, and providing carbon skeletons for biosynthesis, the process is highly regulated. One key enzyme in this control is phosphofructokinase, which in bacteria is shown to comprise two similar roles, one involved in ATP binding and the other housing both the substrate-binding site and the allosteric site. The latter is a regulatory site affecting enzyme activity which is distinct from the active site. Phosphofructokinase is in itself under tight regulation by the ratio of ATP/AMP in the cell. High levels of ATP inhibit phosphofructokinase by attachment of the molecule to its allosteric site and reducing its affinity for fructose 6-phosphate, while AMP can reverse the inhibitory effect of ATP. Levels of citrate, an early intermediate in the citric acid cycle, also affect the activity of phosphofructokinase, as the compound enhances the inhibitory effect of ATP. This allows a negative feedback control of glycolysis, whereby abundant biosynthetic precursors inhibit glycolysis which is a source of carbon skeletons for biosynthesis.

E. coli has two phosphofructokinase activities. Pfk-1, an allosteric enzyme (82) representing 90% of the phosphofructokinase activity in the wild type strain, and Pfk-2, apparently not allosteric and which represent the remaining enzymatic activity in the cell (211). Pfk-1 is coded by the *pfkA* gene (1057), while the expression of the minor phosphofructokinase Pfk-2 is determined by the *pfkB* locus (211). The two phosphofructokinase activities are the reason why *pfkA* mutants, even deletions, still contain phosphofructokinase activity (884). These levels are however insufficient for normal growth on sugars, and such mutants were found to grow slowly or not at all on carbon sources whose major route of degradation is via fructose-6-phosphate (31, 726).

A similar situation is possible in *S. marcescens* Db11, where by mutant N6-B2 which has a transposon insertion in the *pfkA* genes was able to be isolated and grow on TSA and TSB. However as it was the case with *E. coli pfkA* mutants, N6-B2 grew much slower than the wild type and had a statistically significantly lower growth rate value than the parent strain (Table 3.11.). The mutant grew nearly 40% more slowly than the wild type as deduced from growth rate values. The mutant also exhibited an increased susceptibility phenotype whether it was for biocides or antibiotics. Agar MIC tests indicated that the *pfkA* mutant was more sensitive to triclosan, *ortho*-phthalaldehyde, and cetylpyridinium chloride than the wild type. Antibiotic susceptibility profiles showed that the mutant had increased sensitivity to 6 out of the 10 antibiotics tested. These were meropenem, ciprofloxacin, tobramycin, azithromycin, piperacillin and amikacin.

Given the crucial role phosphofructokinase plays in catabolism, maintaining energy balance, and biosynthesis in the cell, the mutant phenotype is not surprising. As mentioned above, although phosphofructokinase is involved in carbohydrate catabolism, the process is linked to many biosynthesis pathways within the cell. Hence, a phosphofructokinase deficient mutant could suffer from nutrients deficiency. This also could explain its slow rate of growth compared with the wild type. There have been many reported linking nutrients limitation or deficiency, growth rate, and antimicrobial susceptibility. This is because growth limitation by different nutrients gives rise to cells with reduced growth rates and coincidentally radically altered envelopes (114, 116, 266, 447, 576).

In Gram-negative bacteria susceptibility changes have been shown to be associated with modifications of both the outer and cytoplasmic membranes (266, 628, 774). These changes have been widely reported to influence greatly susceptibility to antimicrobial agents (116, 344) and to antibiotics (115, 222) for a wide range of organisms (112, 338, 447, 709). Cell size is also altered as a function of growth rate (240, 340, 562, 826, 977), which in turn affects the cell surface area/volume ratio, and alters the outer surface characteristics of the cell. Variation in cell size has been shown to change the exclusion resistance to antimicrobial agents (977), and the susceptibility to drugs which bind strongly to or act at the cell envelope such as polymyxin and tetracycline (113).

Agents which interact with, or specifically act on, the cell envelope are particularly relevant in this context. For instance alteration in susceptibility as result of changes in surface properties related to nutrients limitation and growth rate were reported for biguanides (119, 474, 549), gentamicin (810), and polymyxin (291, 1166), all of which interact directly with specific envelope lipids. Gilbert and Brown (339) showed that *E. coli* grown in carbon-limited environment, was particularly susceptible to the actions of substituted phenols and 2-phenoxyethanol. Similarly, Wright and Gilbert demonstrated that sensitivity of *E. coli* to chlorhexidine and to *n*-alkyltrimethylammonium bromides was greatly influenced by both growth rate and the type of limited nutrients in the media (1165, 1166). They argued that alterations in envelope lipophilicity through changes in the growth rate and nutrients limitation could be the reason for the observed changes.

In relation to antibiotics, β-lactams are highly affected by nutrients limitation and growth rate changes. This is because the expression of penicillin-binding proteins is tightly correlated to growth rate (115, 199, 1076). For example, the β-lactam CGP 17520 which acts on penicillin-binding proteins (111), is particularly effective against slowly growing cultures (199, 1077). Similar to the β-lactams, the aminoglycoside antibiotics tobramycin and streptomycin (733, 857) as well as quinolones agents such as ciprofloxacin, are all growth rate dependent in their action (1190, 1191). The activity of polymyxin is also governed by nutrient limitation and growth rate (245, 1166). For instance, when the effect of polymyxin B on *E. coli* growing at various

nutrients limitation was assessed, it was found that the actions of the agent were different depending on the nutrient lacking from the media (1166). Similar observations were noted with *P. aeruginosa.* When the organism was grown in magnesium-depleted media, the bacterium lost susceptibility to EDTA and polymyxin B, depending on other metal cations present in the medium (114). The organism also became particularly susceptible to the above two agents as its growth rate increased (291).

From the above, it is possible to speculate a chain of thoughts that would explain the phenotype seen in the phosphofructokinase-deficient mutant N6-B2. Transposon insertion would have inactivated the *pfkA* gene, however the enzyme would have still been produced at low level as it is the case in *E. coli* mutants (884). These levels are not sufficient for normal growth and this could be the cause of the low growth rate seen in the mutant. Deficiency in such a key enzyme would also mean that the cell is nutrients deficient as the levels of key intermediates in many biosynthetic pathways are altered. Changes in growth rate and nutrients deficiency would have altered cell size, and both cytoplasmic and outer membrane characteristics of the cell. This in turn would have affected the antimicrobial susceptibility of the mutant. The mutant became more sensitive to biocides that act on the membrane such as cetylpyridinium chloride. Changes in surface layer would also have made it easier for other biocides such as triclosan and *ortho*-phthalaldehyde to gain access to the cell and exert their action. Triclosan is known to inhibit lipid biosynthesis (685), while glycolysis intermediate compounds fuel the former process. This could also explain the increase sensitivity of the mutant to triclosan.

Antibiotics susceptibility of the mutant could also be explained based on the relationship between growth rate, nutrients limitation and antimicrobial sensitivity. The mutant showed increased susceptibility to meropenem, ciprofloxacin, tobramycin, azithromycin, piperacillin and amikacin. As mentioned above, aminoglycosides such as tobramycin are growth rate dependent in their actions, and so are some quinolone agents such as ciprofloxacin. In addition, since the expression of penicillin-binding proteins is highly growth rate dependent, the susceptibility to β-lactam antibiotic such as meropenem, azithromycin and piperacillin could also be affected. Further investigations are needed to explore the above speculations. These

could include complementation of the mutant, detection of phosphofructokinase activity using protein profiling, and microscopy to determine changes in cell size.

3.5.2.2.10 Putative DeoR family transcriptional regulator gene

Transposon insertions in nine mutants (10-E7, 3-F2, 18-A3, 9-D5, 3-A4, 11-B8, 7-C10, N2-B3, and N2-F3) were all located in a single ORF encoding a putative DeoR family transcriptional regulator. This family of prokaryotic regulators is named after the *E. coli* DeoR repressor and is present in a variety of bacterial organisms, ranging from Gram-positives such as *S. mutans* to Gram-negatives such as *P. aeruginosa* (265). The *E. coli* family of DeoR regulators has at least 14 members, which usually act as repressors in sugar metabolism (814). The *deo* operon in *E. coli* consists of four structural genes encoding ribonucleoside and deoxyribonucleoside catabolizing enzymes, which are controlled positively by the cAMP/CRP activator complex and negatively by the DeoR and CytR repressors (736, 995). These genes encode deoxyriboaldolase (*deoC*), phosphodeoxyribomutase (*deoB*), and thymidine phosphorylase (*deoA*), and purine nucleoside phosphorylase (*deoD)* (396).

Munch-Petersen and Mygind (735) demonstrated that in *E. coli* K-12, the nucleoside catabolizing enzymes are located inside the permeability barrier of the cell. DeoR also regulate the expression of NupG, a nucleoside transpoter protein (735), as well as Tsx a channel-forming protein (101). Given that DeoR regulates not only sugar metabolism but also the expression of a number of membrane barrier enzymes, transporters and channel-forming proteins, disruption in this gene would not only affect for instance the deoxyribonucleoside catabolism but also alter the membrane permeability. This may in turn alter the antimicrobial susceptibility of the cell.

Changes in antimicrobial susceptibility in the DeoR-deficient mutant could not only be a result of altered sugar metabolism and related enzymes but also as a result of its global regulatory effect. Indeed DeoR-type family of transcriptional regulators has been shown to effect the expression or a variety of different genes. Elgrably-Weiss *et al.* (265) reported that DeoT, an *E. coli* protein with homology to DeoR-type family of transcriptional regulators, acts as a global regulator, repressing the expression of a number of genes involved in a variety of metabolic pathways including transport of

maltose, fatty acid β-oxidation, and peptide degradation. In the context of antimicrobial susceptibility, Ramos-Aires *et al.* (852) reported that GlmR, a transcriptional regulator with amino acid sequence similarity to DeoR-family repressors, was able to affect susceptibility to a large variety of antibiotics in *P. aeruginosa*. They found that a transposon mutant 19A, which was hypersusceptible to a wide range of antibiotics including aminoglycosides, β-lactams, fluoroquinolones, colistin, erythromycin, rifampin, and glycopeptides, had transposon insertion in the *glmR* gene coding for GlmR repressor.

Because of the influence of DeoR on gene expression and the possibility of polar effect from the mutation on genes downstream from the disrupted putative *deoR*, it was worth exploring the genes surrounding the site of transposon insertion in the mutants. The *deoR* locus was surrounded by genes coding the following putative proteins:

1) Glyoxalase: In living organisms, detoxification of α-ketoaldehydes substrates such as methylglyoxal, a mutagenic and cytotoxic compound mainly formed as a by-product of glycolysis and amino acid catabolism, is achieved by the glyoxalase system (1060). α-Ketoaldehydes substrates are cytotoxic and mutagenic because they interact with functional groups such as amines and thiols, hence covalently modifying DNA, RNA, and proteins in cellular systems (512, 737, 943). Many biocides including heavy metals, aldehydes and oxidizing agents act by interaction with functional groups especially thiols (section 1.3). Hence a mutant with altered or deficient glyoxalase enzymes, unable to efficiently detoxify α-ketoaldehydes substrates, would probably be more susceptible to biocides than the wild type.

2) LysR family transcriptional regulator: The LysR-type transcriptional regulators were first described by Henikoff *et al.* (427), and are thought to constitute the largest family of prokaryotic DNA binding proteins, also present in diverse archaeal genera and algal chloroplasts (953). LysR-type transcriptional regulators are similarly sized molecules (300–350 amino acids) that activate the transcription of linked target genes or unlinked regulons encoding extremely diverse functions including nitrogen fixation, oxidative stress response and bacterial virulence (953). Given the influence of LysR regulators on other genes, alterations in the expression of these proteins are

likely to influence a number of cellular processes. In this study, the gene encoding the putative divalent heavy-metal cations transporter involved in inorganic ion transport and metabolism, and that encoding a putative heat shock protein DnaJ homologue, both located downstream of the LysR coding gene could be potentially affected by LysR expression.

3) Pirin: The protein pirin is widely found and conserved from prokaryotic microorganisms, fungi, and plants to mammals (1137). Pirin is involved in a variety of biological processes, and was shown to act as transcriptional cofactor or an apoptosis-related protein in mammals (226). The protein was shown to be involved in seed germination and seedling development in *Arabidopsis* (583) and programmed cell death in tomato (783). Few prokaryotic pirin orthologs have been characterised, however Hihara *et al.* (436) showed that in cyanobacteria pirin was induced under stress conditions and was negatively regulated by a LysR family of transcriptional regulator encoded by *pirR* located immediately upstream of the pirin gene. PirR was reported to also repress expression of closely located ORFs in addition to the pirin gene and *priR* itself (436). In *E. coli* the protein was shown to act as a quercetinase involved in the degradation of the antioxidant quercetin (3). Recently, Soo *et al.* (997) demonstrated that the *S. marcescens* pirin ($pirin_{Sm}$) gene encoding an ortholog of pirin protein played a regulatory role in the process of pyruvate catabolism to acetyl coenzyme A through the interaction with the pyruvate dehydrogenase E1 subunit and in inhibiting the enzyme complex activity. Investigators observed significant increase in the activity of pyruvate dehydrogenase complex and in the concentration of ATP and NADH/NAD^+ ratio in a *S. marcescens* mutant with disrupted ($pirin_{Sm}$) gene (997). Hence it is clear that pirin protein has a wide range of functions and potentially able to influence many metabolic and energy process in the cell as well as affect the expression of other genes.

4) Isochorismatase: The enzyme isochorismatase belongs to the hydrolases family. It is also known as 2,3 dihydro-2,3 dihydroxybenzoate synthase or isochorismate pyruvate-hydrolase and catalyses the conversion of isochorismate, in the presence of water, to 2,3-dihydroxybenzoate and pyruvate (1184). The enzyme is involved in the biosynthesis of siderophore group nonribosomal peptides, which are important in the acquisition of iron from the environment. Iron is an essential nutrient for virtually all

microorganisms because it is a cofactor for several electron-transport proteins involved in vital life processes like aerobic and anaerobic ATP biosynthesis. Iron is also important for invading pathogens, as it is considered essential for establishing infection (1053). The bioavailability of iron, which exists predominantly in its ferric form in aerobic environments, is very low, and micro-organisms used biosynthesis and excretion of high-affinity iron chelators known as siderophores to get hold of the available iron (249, 1053). Many siderophores are polypeptides that are biosynthesised by members of the nonribosomal peptide synthetase multienzyme family (200). As iron is an important nutrient, and nutrient limitation has a great influence on antimicrobial sensitivity (section 3.5.2.2.9.), a mutant with a disrupted isochorismatase encoding gene, would be expected to have altered biocide and antibiotic susceptibility.

All but three of the *deoR* mutants showed increased sensitivity to cetylpyridinium chloride, and six of the mutants were sensitive to *ortho*-phthalaldehyde. Mutants 3-A4, N2-B3, 7-C10, and N2-F3 were also sensitive to triclosan, and mutants 18-A3, 9-D5 and N2-B3 all showed increased sensitivity to chlorhexidine diacetate. Interestingly, mutants 3-A4 and N2-F3 expressed a resistant phenotype to the latter biocide. Antibiotic susceptibility profiles showed that out of the 9 *deoR* mutants 6 had increased sensitivity to azithromycin and a similar number to meropenem. Amikacin resistance was seen in 6 mutants, and mutant 18-A3 was particularly interesting as it developed resistance to 8 out of the 10 antibiotics tested

The reason why so many mutants had transposon insertions into the *deoR* gene is not clear, however the diverse biocide and antibiotic phenotypes seen for the mutants could be explained as follows. From the information above, it is clear that *deoR* controls the expression of a large number of genes involved in many cellular processes including genes for sugar metabolism, membrane barrier enzymes, transporters and channel-forming proteins. In addition, DeoR could also influence the expression of genes downstream from its location encoding a putative glyoxalase, pirin, and isochorismatase, all have the potential to influence antimicrobial susceptibility. As a result of its long distance regulation (213), DeoR could also regulate the expression of genes even further downstream from the ones mentioned above. Moreover, possible polar effects of the mutations in *deoR*, could effect the

expression of the LysR regulator, which inturn would alter the expression of genes downstream from its location, such as that of the putative heavy-metal cations transporter and putative heat shock protein DnaJ homologue, both of which have the potential to alter antimicrobial susceptibility. As a result, the location of transposon insertion in such a highly regulatory region of the *S. marcescens* Db11 genome, containing a number of genes which have the potential to affect many cellular process and antimicrobial susceptibility, it is not surprising that the *deoR* mutants possessed such a varied phenotype. In addition to this, the transposon insertions in the putative *deoR* were not in the same location in all mutants (Figure 3.21), which could also explain the different phenotypes.

3.5.2.2.11 Putative carbamoyl-phosphate synthase large subunit gene (*carB*)

Transposon insertion in mutants 8-C7 was located within *carB* encoding a putative carbamoyl-phosphate synthase large subunit. The latter is part of a two subunit enzyme, carbamoyl-phosphate synthase, which catalyzes the formation of carbamoyl phosphate, a common intermediate in both arginine and pyrimidine biosynthesis pathways (825). As mentioned above, bacterial carbamoyl-phosphate synthase is a heterodimer of small and large subunits. The small chain encoded by *carA* (827), promotes the hydrolysis of glutamine to ammonia, which is used by the large chain, encoded by *carB* (776), to synthesise carbamoyl phosphate. The enzyme is therefore involved in both amino acid and nucleic acid biosynthesis, and transposon insertion in *carB* has the potential to disrupt both processes.

Mutant 8-C7 had a complex phenotype in that it was more resistant than the wild type to inhibition of growth by triclosan and chlorhexidine diacetate, however it is more sensitive compared with the wild type to the killing effect of the two biocides. Antibiotic susceptibility tests indicated that the mutant showed increased resistance to the two aminoglycosides amikacin and tobramycin but was more susceptible than the wild type to meropenem. Interference in key cellular processes such as amino and nucleic acid biosynthesis has the potential to affect the mutant fitness, growth, protein synthesis, and tolerance to antimicrobial agents. All these could contribute to the increased killing effect of biocides on the mutant. It is interesting however that the mutant became more resistant to the growth inhibitory action of triclosan and

chlorhexidine diacetate as well as to the two aminoglycosides, known to act at the protein in the cell. This suggest that there might be other factors involved in the development of this phenotype, including possibility of other genes affected by the mutation such as those for efflux. Further studies are needed to investigate this matter, including complementation of the mutant.

3.6 CONCLUSION

This study set out to determine the molecular basis of biocide resistance and susceptibility in *Serratia* using the model strain *S. marcescens* Db11. The possible link between biocide and antibiotic susceptibility was also to be explored. Four biocides (triclosan, chlorhexidine diacetate, cetylpyridinium chloride, and *ortho*-phthalaldehyde), and 10 antibiotics (amikacin, azithromycin, chloramphenicol, ciprofloxacin, tobramycin, trimethoprim/sulfamethoxazole, meropenem, ceftazidime, imipenem, and piperacillin) were used to investigate the above. The main conclusions from this study were:

- *S. marcescens* Db11 was shown to be more effected by surface-active agents such as chlorhexidine and cetylpyridium chloride than by the phenolic compound triclosan or the aldehyde *ortho*-phthalaldehyde.

- Db11 was interesting in that unlike many other reported *S. marcescens* strains, it was resistant to aminoglycosides and sensitive to β-lactams.

- Results from this study and reported ones in literature suggested that strain Db11 is relatively resistant to antimicrobial agents compared to Gram-positives such as *S. aureus* and to related Gram-negatives such as *E. coli*. In fact the strain had levels of susceptibility in the range to those of some mycobacteria and *P. aeruginosa*. One exception was in relation to triclosan where only *P. aeruginosa*, known to have triclosan efflux systems, had comparable degrees of resistance to those of Db11, while *E. coli* and mycobacterial strains were much more sensitive to the agent than Db11.

- It can be speculate that the relative resistance to antimicrobial agents seen in Db11 could be at least in part a result of its outer membrane characteristics. The organism contains a number of multidrug efflux pumps and porins, which along with its other outer membrane components has an impact on its antimicrobial susceptibility.

- Random transposon mutagenesis using the mini-Tn*5*Km2 transposon produced a number of mutants with altered biocide sensitivity compared with the wild type. However, biocides agar MICs demonstrated that changes in biocides susceptibility of the mutants tested were relatively small, although these changes were reproducible. This is probably a reflection of the multi-target action of biocides in bacteria.

- Altered antibiotic susceptibility was also evident in the mutants. Given the similarities in the mode of action of biocides and antibiotics and the way they enter the bacterial cell, this is not surprising.

- The locations of transposon insertion in all but 2 of the 26 biocide mutants examined were determined, and 14 putative genes coding for putative proteins with varying functions were found to be disrupted. These functions included anabolism and catabolism, gene regulation, cell envelope biosynthesis, porin, energy production and virulence.

- Complementation in one mutant disrupted in it putative nucleoid-associated protein gene (*ndpA*) successfully restored the wild type phenotype.

It is clear that complementation of all the remaining mutants is necessary to confirm the link between the antimicrobial sensitivity of the mutants and their respective disrupted genes. In addition, it is worth noting that only one strain, Db11, was investigated in this work and study of a larger pool of *S. marcescens* strains is needed to confirm the above conclusions. Nevertheless, this study has given an insight into the levels of antimicrobial sensitivity of *S. marcescens* Db11 and into the molecular basis of biocide and antibiotic resistance and susceptibility in this strain.

3.7 FUTURE WORK

- This work investigated biocide and antibiotic susceptibility in one strain of *S. marcescens*, Db11. It is therefore necessary to study a larger number of strains and isolates to be able to arrive at generalised conclusions about the level of resistance of *S. marcescens* to antimicrobial agents compared with other Gram-negatives, Gram-positives or mycobacteria.

- Complementation of all biocide mutants generated in this study is necessary to confirm that the disrupted genes are connected with the changes in phenotype observed. Introducing a functional copy of the disrupted genes into the mutants should restore the wild type phenotype, proving that these genes play a role in antimicrobial susceptibility. Moreover, site directed mutagenesis of genes downstream from the transposon insertions in the mutants may also be necessary in cases where the disrupted gene is a regulatory gene or in instances where a polar effect of the mutation is suspected.

- Analysis of the outer membrane characteristics of the biocide mutants including their proteins content, lipopolysaccharide structure and their permeability to determine whether there is detectable changes in the transposon mutants compared with the wild type would be beneficial. In this context, electron microscopy such as scanning electron micrographs and transmission electron micrographs could help determine whether cytological changes have taken place in the mutant including changes in membrane structure and cell size.

- Studying genes known or suspected to be involved in biocide resistance or susceptibility is also an option. These can be identified from literature and with the help of the published *S. marcescens* Db11 genome sequence. For example, protein similarity searches against the translated *S. marcescens* Db11 genome sequence (www.sanger.ac.uk) for a number of known multidrug transporters and efflux systems revealed potential antimicrobial resistance

proteins. The MF family drug transporters EmrB, EmD and MdfA/Cmr from *E. coli* showed respectively 75%, 55% and 70% amino acid sequence similarities with *S. marcescens* Db11 putative proteins. In addition, proteins AcrA, AcrB, and TolC components of the well characterised AcrAB-TolC multidrug efflux system in *E. coli*, showed respectively 70%, 79%, and 70% amino acid sequence similarities with *S. marcescens* Db11 putative proteins. This suggests that an AcrAB-TolC system homologue may be present in *S. marcescens* Db11 contributing to antimicrobial resistance in this strain.

Similarly, putative *S. marcescens* Db11 proteins, showed 60%, 68% and 43% amino acid sequence similarities with MexA, MexB, and OprM, proteins respectively of the *P. aeruginosa* MexAB-OprM multidrug efflux system. These results suggest that homologues of at least some of the above drug resistance proteins may exist in *S. marcescens* Db11. Indeed, according to the genome sequence of *S. marcescens* Db11 (ftp://ftp.sanger.ac.uk/pub/pathogens/sm/), many other multidrug efflux pumps are expected to be present without any physiological characterisation. Genes encoding for potential efflux systems could be disrupted using site directed mutagenesis and the resulting effect characterised in *S. marcescens* Db11. Similarly, the same genes could be cloned into a drug hypersensitive bacterial strain such as *E. coli* KAM32 (158) and checked for increase antimicrobial resistance.

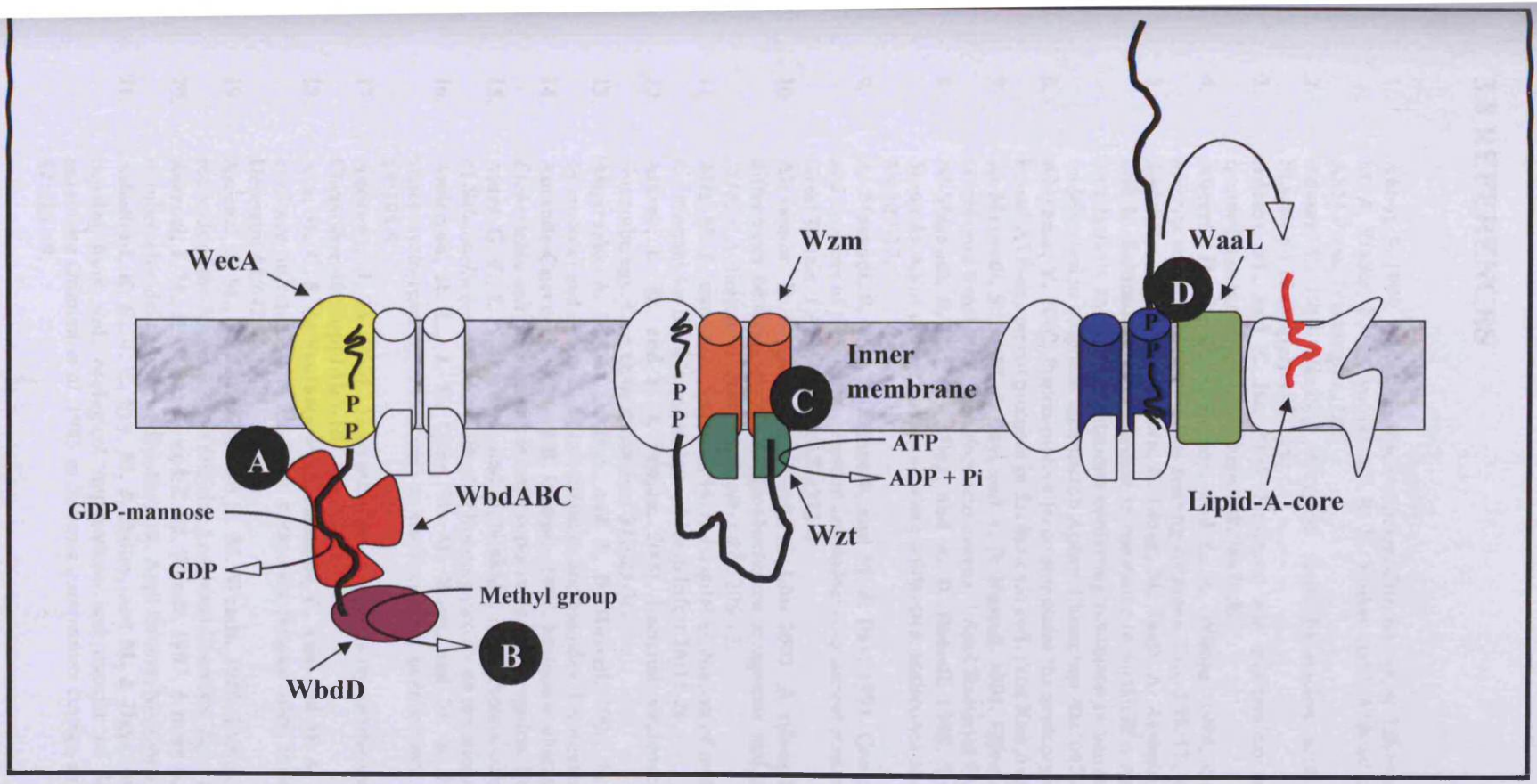


Figure 3.30. Model for O-antigen biosynthesis in *E. coli* O8. Adapted from Cuthbertson *et al.* (206).

Synthesis of the O-antigen using the ATP-binding cassette (ABC)-transporter-dependent pathway is as follows:
Step A: synthesis is initiated by the transfer of *N*-acetylglucosamine-1-phosphate by WecA. Glycosyltransferases WbdABC then synthesize the polymannan O-antigenic polysaccharide through addition of mannose residues from the guanosinediphosphate (GDP)-mannose donor to the nonreducing terminus. Step B: addition of a methyl group by WbdD causes termination of polymannan growth. Step C: the ABC-transporter, consisting of Wzm and Wzt, relocate the finished polymer to the periplasmic side of the inner membrane. Step D: the O-antigenic polysaccharide is then transferred to the lipid A core molecule in a reaction involving WaaL.

3.8 REFERENCES

1. **Abbot, S.** 1999. *Klebsiella, Enterobacter, Citrobacter,* and *Serratia. In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. ASM Press, Washington, DC.
2. **Adams, E.** 1968. Binding of crystal violet by nucleic acids of *Escherichia coli.* J Pharm Pharmacol **20 (Suppl):**18S-22S.
3. **Adams, M., and Z. Jia.** 2005. Structural and biochemical analysis reveal pirins to possess quercetinase activity. J Biol Chem **280:**28675-82.
4. **Ahearn, D. G., C. A. Penley, and L. A. Wilson.** 1984. Growth and survival of *Serratia marcescens* in hard contact lens wetting solutions. Clao J **10:**172-4.
5. **Akimitsu, N., H. Hamamoto, R. Inoue, M. Shoji, A. Akamine, K. Takemori, N. Hamasaki, and K. Sekimizu.** 1999. Increase in resistance of methicillin-resistant *Staphylococcus aureus* to beta-lactams caused by mutations conferring resistance to benzalkonium chloride, a disinfectant widely used in hospitals. Antimicrob Agents Chemother **43:**3042-3.
6. **Akiyama, Y.** 2002. Proton-motive force stimulates the proteolytic activity of FtsH, a membrane-bound ATP-dependent protease in *Escherichia coli.* Proc Natl Acad Sci USA **99:**8066-71.
7. **Al-Masaudi, S. B., M. J. Day, and A. D. Russell.** 1991. Effect of some antibiotics and biocides on plasmid transfer in *Staphylococcus aureus.* J Appl Bacteriol **71:**239-43.
8. **Al-Masaudi, S. B., M. J. Day, and A. D. Russell.** 1988. Sensitivity of methicillin-resistant *Staphylococcus aureus* strains to some antibiotics, antiseptics and disinfectants. J Appl Bacteriol **65:**329-37.
9. **Al-Masaudi, S. B., A. D. Russell, and M. J. Day.** 1991. Comparative sensitivity to antibiotics and biocides of methicillin-resistant *Staphylococcus aureus* strains isolated from Saudi Arabia and Great Britain. J Appl Bacteriol **71:**331-8.
10. **Alexander, D. C., J. R. Jones, and J. Liu.** 2003. A rifampin-hypersensitive mutant reveals differences between strains of *Mycobacterium smegmatis* and presence of a novel transposon, *IS1623.* Antimicrob Agents Chemother **47:**3208-13.
11. **Alfa, M. J., and D. L. Sitter.** 1994. In-hospital evaluation of *ortho*phthalaldehyde as a high level disinfectant for flexible endoscopes. J Hosp Infect **26:**15-26.
12. **Alksne, L. E., and S. J. Projan.** 2000. Bacterial virulence as a target for antimicrobial chemotherapy. Curr Opin Biotechnol **11:**625-36.
13. **Alqurashi, A. M., M. J. Day, and A. D. Russell.** 1996. Susceptibility of some strains of enterococci and streptococci to antibiotics and biocides. J Antimicrob Chemother **38:**745.
14. **Amabile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res **19:**4479-84.
15. **Ames, G. F., E. N. Spudich, and H. Nikaido.** 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. J Bacteriol **117:**406-16.
16. **Anderson, R. L., J. H. Carr, W. W. Bond, and M. S. Favero.** 1997. Susceptibility of vancomycin-resistant enterococci to environmental disinfectants. Infect Control Hosp Epidemiol **18:**195-9.
17. **Andrews, J. M.** 2001. Determination of minimum inhibitory concentrations. J Antimicrob Chemother **48 (Suppl 1):**5-16.
18. **Ara, M., C. S. de Santamaria, P. Zaballos, C. Yus, and M. A. Lezcano.** 2003. *Mycobacterium chelonae* infection with multiple cutaneous lesions after treatment with acupuncture. Int J Dermatol **42:**642-4.
19. **Ascenzi, J. M., R. J. Ezzell, and T. M. Wendt.** 1986. Evaluation of carriers used in the test methods of the Association of Official Analytical Chemists. Appl Environ Microbiol **51:**91-4.
20. **Ascenzi, J. M., R. J. Ezzell, and T. M. Wendt.** 1987. A more accurate method for measurement of tuberculocidal activity of disinfectants. Appl Environ Microbiol **53:**2189-92.
21. **Ashelford, K. E., J. C. Fry, M. J. Bailey, and M. J. Day.** 2002. Characterization of *Serratia* isolates from soil, ecological implications and transfer of *Serratia proteamaculans* subsp. *quinovora* Grimont *et al.* 1983 to *Serratia quinivorans* corrig., sp. nov. Int J Syst Evol Microbiol **52:**2281-9.

22. **Assadian, O., A. Berger, C. Aspock, S. Mustafa, C. Kohlhauser, and A. M. Hirschl.** 2002. Nosocomial outbreak of *Serratia marcescens* in a neonatal intensive care unit. Infect Control Hosp Epidemiol **23:**457-61.
23. **Aucken, H. M., and T. L. Pitt.** 1998. Antibiotic resistance and putative virulence factors of *Serratia marcescens* with respect to O and K serotypes. J Med Microbiol **47:**1105-13.
24. **Axon, A. T., and P. B. Cotton.** 1983. Endoscopy and infection. Gut **24:**1064-6.
25. **Aydin, S., Z. Erman, and Ö. C. Bilgin.** 2001. Investigations of *Serratia liquefaciens* infection in rainbow trout (Oncorhynchus mykiss Walbaum). Turk J Vet Anim Sci **25:**643-50.
26. **Ayliffe, G. A., D. Coates, and P. N. Hoffmann.** 1993. Chemical Disinfection in Hospitals. Public Health Laboratory, London.
27. **Ayres, H. M., J. R. Furr, and A. D. Russell.** 1999. Effect of permeabilizers on antibiotic sensitivity of *Pseudomonas aeruginosa*. Lett Appl Microbiol **28:**13-6.
28. **Azachi, M., Y. Henis, R. Shapira, and A. Oren.** 1996. The role of the outer membrane in formaldehyde tolerance in *Escherichia coli* VU3695 and *Halomonas sp.* MAC. Microbiology **142:**1249-54.
29. **Azadian, B. S., A. Beck, J. R. Curtis, L. E. Cherrington, P. E. Gower, M. Phillips, J. B. Eastwood, and J. Nicholls.** 1981. Disseminated infection with *Mycobacterium chelonei* in a haemodialysis patient. Tubercle **62:**281-4.
30. **Azam, T. A., and A. Ishihama.** 1999. Twelve species of the nucleoid-associated protein from *Escherichia coli*. Sequence recognition specificity and DNA binding affinity. J Biol Chem **274:**33105-13.
31. **Babul, J.** 1978. Phosphofructokinases from *Escherichia coli*. Purification and characterization of the nonallosteric isozyme. J Biol Chem **253:**4350-5.
32. **Baillie, L.** 1987. Chlorhexidine resistance among bacteria isolated from urine of catheterized patients. J Hosp Infect **10:**83-6.
33. **Baillie, L. W., J. J. Wade, and M. W. Casewell.** 1992. Chlorhexidine sensitivity of *Enterococcus faecium* resistant to vancomycin, high levels of gentamicin, or both. J Hosp Infect **20:**127-8.
34. **Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. Salmond, G. S. Stewart, and P. Williams.** 1992. *N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*. Biochem J **288:**997-1004.
35. **Bakken, J. S., C. C. Sanders, and K. S. Thomson.** 1987. Selective ceftazidime resistance in *Escherichia coli*: association with changes in outer membrane protein. J Infect Dis **155:**1220-5.
36. **Baldock, C., G. J. de Boer, J. B. Rafferty, A. R. Stuitje, and D. W. Rice.** 1998. Mechanism of action of diazaborines. Biochem Pharmacol **55:**1541-9.
37. **Baldry, M. G.** 1983. The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. J Appl Bacteriol **54:**417-23.
38. **Ball, A. P., D. McGhie, and A. M. Geddes.** 1977. *Serratia marcescens* in a general hospital. Q J Med **46:**63-71.
39. **Balsalobre, C., J. Johansson, B. E. Uhlin, A. Juarez, and F. J. Munoa.** 1999. Alterations in protein expression caused by the *hha* mutation in *Escherichia coli*: influence of growth medium osmolarity. J Bacteriol **181:**3018-24.
40. **Bamber, A. I., and T. J. Neal.** 1999. An assessment of triclosan susceptibility in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*. J Hosp Infect **41:**107-9.
41. **Banerjee, S. K., K. Bhatt, P. Misra, and P. K. Chakraborti.** 2000. Involvement of a natural transport system in the process of efflux-mediated drug resistance in *Mycobacterium smegmatis*. Mol Gen Genet **262:**949-56.
42. **Bar-Ness, R., and M. Rosenberg.** 1989. Putative role of a 70 kDa outer-surface protein in promoting cell-surface hydrophobicity of *Serratia marcescens* RZ. J Gen Microbiol **135:**2277-81.
43. **Barker, K., H. Fan, C. Carroll, G. Kaplan, J. Barker, W. Hellmann, and Z. A. Cohn.** 1996. Nonadherent cultures of human monocytes kill *Mycobacterium smegmatis*, but adherent cultures do not. Infect Immun **64:**428-33.
44. **Barksdale, L., and K. S. Kim.** 1977. *Mycobacterium*. Bacteriol Rev **41:**217-372.
45. **Barkvoll, P., and G. Rolla.** 1994. Triclosan protects the skin against dermatitis caused by sodium lauryl sulphate exposure. J Clin Periodontol **21:**717-9.

46. **Barnes, P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin.** 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. J Immunol **149:**541-7.
47. **Barnum, D. A., E. L. Thackeray, and N. A. Fish.** 1958. An outbreak of mastitis caused by *Serratia marcesens*. Can J Comp Med **22:**392.
48. **Barrett-Bee, K., L. Newboult, and S. Edwards.** 1994. The membrane destabilising action of the antibacterial agent chlorhexidine. FEMS Microbiol Lett **119:**249-53.
49. **Barry, M. A., D. E. Craven, T. A. Goularte, and D. A. Lichtenberg.** 1984. *Serratia marcescens* contamination of antiseptic soap containing triclosan: implications for nosocomial infection. Infect Control **5:**427-30.
50. **Bartzokas, C. A., J. E. Corkill, and T. Makin.** 1987. Evaluation of the skin disinfecting activity and cumulative effect of chlorhexidine and triclosan handwash preparations on hands artificially contaminated with *Serratia marcescens*. Infect Control **8:**163-7.
51. **Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman.** 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Mol Microbiol **9:**773-86.
52. **Bastin, D. A., G. Stevenson, P. K. Brown, A. Haase, and P. R. Reeves.** 1993. Repeat unit polysaccharides of bacteria: a model for polymerization resembling that of ribosomes and fatty acid synthetase, with a novel mechanism for determining chain length. Mol Microbiol **7:**725-34.
53. **Batchelor, R. A., G. E. Haraguchi, R. A. Hull, and S. I. Hull.** 1991. Regulation by a novel protein of the bimodal distribution of lipopolysaccharide in the outer membrane of *Escherichia coli*. J Bacteriol **173:**5699-704.
54. **Baya, A. M., A. E. Toranzo, B. Lupiani, Y. Santos, and F. M. Hetrick.** 1992. *Serratia marcescens*: a potential pathogen for fish. J Fish Dis **15:**15–26.
55. **Beck, E., and E. Bremer.** 1980. Nucleotide sequence of the gene *ompA* coding the outer membrane protein II of *Escherichia coli* K-12. Nucleic Acids Res **8:**3011-27.
56. **Becker, A., K. Niehaus, and A. Puhler.** 1995. Low-molecular-weight succinoglycan is predominantly produced by *Rhizobium meliloti* strains carrying a mutated ExoP protein characterized by a periplasmic N-terminal domain and a missing C-terminal domain. Mol Microbiol **16:**191-203.
57. **Beeson, J. G., P. A. Winstanley, G. I. McFadden, and G. V. Brown.** 2001. New agents to combat malaria. Nat Med **7:**149-50.
58. **Beher, M. G., C. A. Schnaitman, and A. P. Pugsley.** 1980. Major heat-modifiable outer membrane protein in gram-negative bacteria: comparison with the *ompA* protein of *Escherichia coli*. J Bacteriol **143:**906-13.
59. **Behr, H., M. E. Reverdy, C. Mabilat, J. Freney, and J. Fleurette.** 1994. Relationship between the level of minimal inhibitory concentrations of five antiseptics and the presence of *qacA* gene in *Staphylococcus aureus*. Pathol Biol **42:**438-44.
60. **Belaaouaj, A., K. S. Kim, and S. D. Shapiro.** 2000. Degradation of outer membrane protein A in *Escherichia coli* killing by neutrophil elastase. Science **289:**1185-8.
61. **Belser, W. L., and M. I. Bunting.** 1956. Studies on a mechanism providing for genetic transfer in *Serratia marcescens*. J Bacteriol **72:**582-92.
62. **Bengoechea, J. A., E. Pinta, T. Salminen, C. Oertelt, O. Holst, J. Radziejewska-Lebrecht, Z. Piotrowska-Seget, R. Venho, and M. Skurnik.** 2002. Functional characterization of Gne (UDP-*N*-acetylglucosamine-4-epimerase), Wzz (chain length determinant), and Wzy (O-antigen polymerase) of *Yersinia enterocolitica* serotype O:8. J Bacteriol **184:**4277-87.
63. **Benito, H., M. E. Reverdy, C. Mabilat, and J. Fleurette.** 1994. Relationship between the level of minimal inhibitory concentrations of five antiseptics and the presence of *qacA* gene in *Staphylococcus aureus*. Pathol Biol **42:**438-444.
64. **Bennett, J. W., and R. Bentley.** 2000. Seeing red: the story of prodigiosin. Adv Appl Microbiol **47:**1-32.
65. **Bentley, J., L. S. Hyatt, K. Ainley, J. H. Parish, R. B. Herbert, and G. R. White.** 1993. Cloning and sequence analysis of an *Escherichia coli* gene conferring bicyclomycin resistance. Gene **127:**117-20.
66. **Berg, G.** 2000. Diversity of antifungal and plant-associated *Serratia plymuthica* strains. J Appl Microbiol **88:**952-60.

67. **Bergan, T., and A. Lystad.** 1971. Antitubercular action of disinfectants. J Appl Bacteriol **34:**751-6.
68. **Bergey, D. H., F. C. Harrison, R. S. Breed, B. W. Hammer, and F. M. Huntoon.** 1923. Bergey's Manual of Determinative Bacteriology. Williams & Wilkins., Baltimore, USA.
69. **Berlanga, M., N. Ruiz, J. Hernandez-Borrell, T. Montero, and M. Vinas.** 2000. Role of the outer membrane in the accumulation of quinolones by *Serratia marcescens*. Can J Microbiol **46:**716-22.
70. **Besra, G. S., and P. Brennan.** 1994. The glycolipids of mycobacteria, p. 203-32. *In* C. Fenselau (ed.), Mass spectrometry for the characterization of microorganisms. Amer Chem, Washington, DC.
71. **Besra, G. S., and P. Brennan.** 1997. The mycobacterial cell envelope: atarget for novel drugs against tuberculosis. J Pharm Pharmacol. **49:**25-30.
72. **Besra, G. S., and P. J. Brennan.** 1997. The mycobacterial cell wall: biosynthesis of arabinogalactan and lipoarabinomannan. Biochem Soc Trans **25:**845-50.
73. **Besra, G. S., and D. Chatterjee.** 1996. Lipids and carbohydrates of *Mycobacterium tuberculosis*, p. 265-306. *In* B. R. Bloom (ed.), Tuberculosis: Pathogenesis, protection, and control. ASM, Washington, DC.
74. **Best, M., S. A. Sattar, V. S. Springthorpe, and M. E. Kennedy.** 1988. Comparative mycobactericidal efficacy of chemical disinfectants in suspension and carrier tests. Appl Environ Microbiol **54:**2856-8.
75. **Best, M., S. A. Sattar, V. S. Springthorpe, and M. E. Kennedy.** 1990. Efficacies of selected disinfectants against *Mycobacterium tuberculosis*. J Clin Microbiol **28:**2234-9.
76. **Bhargava, H. N., and P. A. Leonard.** 1996. Triclosan: applications and safety. Am J Infect Control **24:**209-18.
77. **Bi, E., K. Dai, S. Subbarao, B. Beall, and J. Lutkenhaus.** 1991. FtsZ and cell division. Res Microbiol **142:**249-52.
78. **Billman-Jacobe, H., R. E. Haites, and R. L. Coppel.** 1999. Characterization of a *Mycobacterium smegmatis* mutant lacking penicillin binding protein 1. Antimicrob Agents Chemother **43:**3011-3.
79. **Billman-Jacobe, H., J. Sloan, and R. L. Coppel.** 1996. Analysis of isoniazid-resistant transposon mutants of *Mycobacterium smegmatis*. FEMS Microbiol Lett **144:**47-52.
80. **Bizio, B.** 1923. Bibl. Ital. G. L. Sci Art **30:**275-295.
81. **Blakley, R. L., and H. A. Barker.** 1964. Cobamide stimulation of the reduction of ribotides to deoxyribotides in *Lactobacillus leichmannii*. Biochem Biophys Res Commun **16:**391-7.
82. **Blangy, D., H. Buc, and J. Monod.** 1968. Kinetics of the allosteric interactions of phosphofructokinase from *Escherichia coli*. J Mol Biol **31:**13-35.
83. **Block, S. S.** 2001. Peroxygen compounds, p. 185–204. *In* S. S. Block (ed.), Disinfection, sterilization and preservation, 5th ed. Lippincott Williams & Wilkins, Philadelphia, USA.
84. **Bobichon, H., and P. Bouchet.** 1987. Action of chlorhexidine on budding *Candida albicans*: scanning and transmission electron microscopic study. Mycopathologia **100:**27-35.
85. **Bolan, G., A. L. Reingold, L. A. Carson, V. A. Silcox, C. L. Woodley, P. S. Hayes, A. W. Hightower, L. McFarland, J. W. Brown, 3rd, N. J. Petersen, and *et al.*** 1985. Infections with *Mycobacterium chelonei* in patients receiving dialysis and using processed hemodialyzers. J Infect Dis **152:**1013-9.
86. **Bollet, C., P. Grimont, M. Gainnier, A. Geissler, J. M. Sainty, and P. De Micco.** 1993. Fatal pneumonia due to *Serratia proteamaculans* subsp. *quinovora*. J Clin Microbiol **31:**444-5.
87. **Borghans, J. G., and J. L. Stanford.** 1973. *Mycobacterium chelone*i in abscesses after injection of diphtheria-pertussis-tetanus-polio vaccine. Am Rev Respir Dis **107:**1-8.
88. **Bosi, C., A. Davin-Regli, R. Charrel, B. Rocca, D. Monnet, and C. Bollet.** 1996. *Serratia marcescens* nosocomial outbreak due to contamination of hexetidine solution. J Hosp Infect **33:**217-24.
89. **Boulton, F. E., S. T. Chapman, and T. H. Walsh.** 1998. Fatal reaction to transfusion of red-cell concentrate contaminated with *Serratia liquefaciens*. Transfus Med **8:**15-8.
90. **Bowman, G. L., W. D. Hueston, G. J. Boner, J. J. Hurley, and J. E. Andreas.** 1986. *Serratia liquefaciens* mastitis in a dairy herd. J Am Vet Med Assoc **189:**913-5.
91. **Bradford, P. A., C. Urban, N. Mariano, S. J. Projan, J. J. Rahal, and K. Bush.** 1997. Imipenem resistance in *Klebsiella pneumoniae* is associated with the combination of ACT-1, a

plasmid-mediated AmpC beta-lactamase, and the foss of an outer membrane protein. Antimicrob Agents Chemother **41:**563-9.

92. **Bradley, C. R., J. R. Babb, and G. A. Ayliffe.** 1995. Evaluation of the steris system 1 peracetic acid endoscope processor. J Hosp Infect **29:**143-51.
93. **Braoudaki, M., and A. C. Hilton.** 2004. Adaptive resistance to biocides in *Salmonella enterica* and *Escherichia coli* O157 and cross-resistance to antimicrobial agents. J Clin Microbiol **42:**73-8.
94. **Braoudaki, M., and A. C. Hilton.** 2004. Low level of cross-resistance between triclosan and antibiotics in *Escherichia coli* K-12 and *E. coli* O55 compared to *E. coli* O157. FEMS Microbiol Lett **235:**305-9.
95. **Braun, G., and S. T. Cole.** 1984. DNA sequence analysis of the *Serratia marcescens ompA* gene: implications for the organisation of an enterobacterial outer membrane protein. Mol Gen Genet **195:**321-8.
96. **Braun, G., and S. T. Cole.** 1983. Molecular characterization of the gene coding for major outer membrane protein OmpA from *Enterobacter aerogenes*. Eur J Biochem **137:**495-500.
97. **Braun, G., and S. T. Cole.** 1982. The nucleotide sequence coding for major outer membrane protein OmpA of *Shigella dysenteriae*. Nucleic Acids Res **10:**2367-78.
98. **Braun, V., and V. Bosch.** 1972. Sequence of the murein-lipoprotein and the attachment site of the lipid. Eur J Biochem **28:**51-69.
99. **Breed, R. S., E. G. D. Murray, and N. R. Smith.** 1957. Bergey's Manual of Determinative Bacteriology. 7 th ed. Williams & Wilkins., Baltimore, USA.
100. **Bremer, E., S. T. Cole, I. Hindennach, U. Henning, E. Beck, C. Kurz, and H. Schaller.** 1982. Export of a protein into the outer membrane of *Escherichia coli* K12. Stable incorporation of the OmpA protein requires less than 193 amino-terminal amino-acid residues. Eur J Biochem **122:**223-31.
101. **Bremer, E., A. Middendorf, J. Martinussen, and P. Valentin-Hansen.** 1990. Analysis of the *tsx* gene, which encodes a nucleoside-specific channel-forming protein (Tsx) in the outer membrane of *Escherichia coli*. Gene **96:**59-65.
102. **Brennan, P.** 1988. *In* C. Ratledge and S. Wilkinson (ed.), Microbial lipids. Academic Press, London, England.
103. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu Rev Biochem **64:**29-63.
104. **Brinton, C. C., Jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Trans N Y Acad Sci **27:**1003-54.
105. **Broadley, S. J., J. R. Furr, P. A. Jenkins, and A. D. Russell.** 1993. Antimycobacterial activity of 'Virkon'. J Hosp Infect **23:**189-97.
106. **Broadley, S. J., P. A. Jenkins, J. R. Furr, and A. D. Russell.** 1991. Antimycobactericidal activity of biocides. Lett Appl Microbiol **13:**118-122.
107. **Broadley, S. J., P. A. Jenkins, J. R. Furr, and A. D. Russell.** 1995. Potentiation of the effects of chlorhexidine diacetate and cetylpyridinium chloride on mycobacteria by ethambutol. J Med Microbiol **43:**458-60.
108. **Brown-Elliott, B. A., and R. J. Wallace, Jr.** 2002. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. Clin Microbiol Rev **15:**716-46.
109. **Brown, B. A., B. Springer, V. A. Steingrube, R. W. Wilson, G. E. Pfyffer, M. J. Garcia, M. C. Menendez, B. Rodriguez-Salgado, K. C. Jost, Jr., S. H. Chiu, and *et al.*** 1999. *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy. Int J Syst Bacteriol **49:**1493-511.
110. **Brown, M. H., I. T. Paulsen, and R. A. Skurray.** 1999. The multidrug efflux protein NorM is a prototype of a new family of transporters. Mol Microbiol **31:**394-5.
111. **Brown, M. R.** 1977. Nutrient depletion and antibiotic susceptibility. J Antimicrob Chemother **3:**198-201.
112. **Brown, M. R., D. G. Allison, and P. Gilbert.** 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J Antimicrob Chemother **22:**777-80.

113. **Brown, M. R., P. J. Collier, and P. Gilbert.** 1990. Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. Antimicrob Agents Chemother **34:**1623-8.
114. **Brown, M. R., and J. Melling.** 1969. Role of divalent cations in the action of polymyxin B and EDTA on *Pseudomonas aeruginosa*. J Gen Microbiol **59:**263-74.
115. **Brown, M. R., and P. Williams.** 1985. Influence of substrate limitation and growth phase on sensitivity to antimicrobial agents. J Antimicrob Chemother **15:**7-14.
116. **Brown, M. R. W.** 1975. The role of the envelope in resistance, p. 71-107. *In* M. R. W. Brown (ed.), Resistance of *Pseudomonas aeruginosa*. John Wiley & Sons, London.
117. **Brown, N. C., Z. N. Canellakis, B. Lundin, P. Reichard, and L. Thelander.** 1969. Ribonucleoside diphosphate reductase. Purification of the two subunits, proteins B1 and B2. Eur J Biochem **9:**561-73.
118. **Brown, N. C., and P. Reichard.** 1969. Ribonucleoside diphosphate reductase. Formation of active and inactive complexes of proteins B1 and B2. J Mol Biol **46:**25-38.
119. **Broxton, P., P. M. Woodcock, F. Heatley, and P. Gilbert.** 1984. Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Escherichia coli*. J Appl Bacteriol **57:**115-24.
120. **Bruck, C. W.** 1991. Role of glutaraldehyde and other liquid chemical sterilants in the processing of new medical devices, p. 376-96. *In* R. F. Morrissey and Y. I. Prokopenko (ed.), Sterilization of medical products, vol.V. Polyscience Publications, Morin Heights, Canada.
121. **Brumfitt, W., S. Dixson, and J. M. Hamilton-Miller.** 1985. Resistance to antiseptics in methicillin and gentamicin resistant *Staphylococcus aureus*. Lancet **1:**1442-3.
122. **Bu'Lock, J. D.** 1961. Intermediary metabolism and antibiotic synthesis. Adv Appl Microbiol **3:**293-342.
123. **Buchanan, R. E.** 1917. Studies in the nomenclature and classification of bacteria. II. The primary subdivisions of the Schizomycetes. J Bacteriol **2:**155-164.
124. **Bucher, G. E.** 1963. Nonsporulating bacterial pathogens, p. 117-47. *In* E. A. Steinhaus (ed.), Insect pathology: An advanced treatise, New York, NY.
125. **Bucher, G. E.** 1959. Bacteria of grasshoppers of Western Canada: III. Frequency of occurrence, pathogenicity. J Insect Pathol **1:**391-405.
126. **Buck, K. M.** 2001. The Effects of Germicides on Microorganisms, Infection control Today Magazine.
127. **Buckley, R., M. W. Cobb, S. Ghurani, N. F. Brock, and R. R. Harford.** 1997. *Mycobacterium fortuitum* infection occurring after a punch biopsy procedure. Pediatr Dermatol **14:**290-2.
128. **Bukau, B., and A. L. Horwich.** 1998. The Hsp70 and Hsp60 chaperone machines. Cell **92:**351-66.
129. **Burrows, L. L., D. Chow, and J. S. Lam.** 1997. *Pseudomonas aeruginosa* B-band O-antigen chain length is modulated by Wzz (Rol). J Bacteriol **179:**1482-9.
130. **Buscher, K. H., W. Cullmann, W. Dick, and W. Opferkuch.** 1987. Imipenem resistance in *Pseudomonas aeruginosa* resulting from diminished expression of an outer membrane protein. Antimicrob Agents Chemother **31:**703-8.
131. **Bush, K.** 1998. Metallo-beta-lactamases: a class apart. Clin Infect Dis **1:**S48-53.
132. **Butler, W. R., K. C. Jost, Jr., and J. O. Kilburn.** 1991. Identification of mycobacteria by high-performance liquid chromatography. J Clin Microbiol **29:**2468-72.
133. **Butler, W. R., L. Thibert, and J. O. Kilburn.** 1992. Identification of *Mycobacterium avium* complex strains and some similar species by high-performance liquid chromatography. J Clin Microbiol **30:**2698-704.
134. **Cabrera, H. A.** 1969. An outbreak of *Serratia marcescens*, and its control. Arch Intern Med **123:**650-5.
135. **Camargo, D., C. Saad, F. Ruiz, M. E. Ramirez, M. Lineros, G. Rodriguez, E. Navarro, B. Pulido, and L. C. Orozco.** 1996. Iatrogenic outbreak of *M. chelonae* skin abscesses. Epidemiol Infect **117:**113-9.
136. **Cambau, E., W. Sougakoff, M. Besson, C. Truffot-Pernot, J. Grosset, and V. Jarlier.** 1994. Selection of a *gyrA* mutant of *Mycobacterium tuberculosis* resistant to fluoroquinolones during treatment with ofloxacin. J Infect Dis **170:**479-83.

137. **Campbell, I. A.** 2000. Management of opportunist mycobacterial infections: Joint Tuberculosis Committee Guidelines 1999. Subcommittee of the Joint Tuberculosis Committee of the British Thoracic Society. Thorax **55:**210-8.
138. **Capponi, M., P. Sureau, and L. Le Minor.** 1956. Bull Soc Pathol Exot Filiales **49:**796-801.
139. **Carbonell, G. V., A. F. Alfieri, A. A. Alfieri, M. C. Vidotto, C. E. Levy, A. L. Darini, and R. M. Yanaguita.** 1997. Detection of cytotoxic activity on Vero cells in clinical isolates of *Serratia marcescens*. Braz J Med Biol Res **30:**1291-8.
140. **Carlson, J., J. A. Fuchs, and J. Messing.** 1984. Primary structure of the *Escherichia coli* ribonucleoside diphosphate reductase operon. Proc Natl Acad Sci USA **81:**4294-7.
141. **Carmona, M., C. Balsalobre, F. Munoa, M. Mourino, Y. Jubete, F. De la Cruz, and A. Juarez.** 1993. *Escherichia coli hha* mutants, DNA supercoiling and expression of the haemolysin genes from the recombinant plasmid pANN202-312. Mol Microbiol **9:**1011-8.
142. **Carson, L. A., L. B. Cusick, L. A. Bland, and M. S. Favero.** 1988. Efficacy of chemical dosing methods for isolating nontuberculous mycobacteria from water supplies of dialysis centers. Appl Environ Microbiol **54:**1756-60.
143. **Carson, L. A., N. J. Petersen, M. S. Favero, and S. M. Aguero.** 1978. Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants. Appl Environ Microbiol **36:**839-46.
144. **Castellani, A., and A. J. Chalmers.** 1919. Manual of tropical medicine, 3rd ed, New York.
145. **Cecchini, G., I. Schroder, R. P. Gunsalus, and E. Maklashina.** 2002. Succinate dehydrogenase and fumarate reductase from *Escherichia coli*. Biochim Biophys Acta **1553:**140-57.
146. **Champion, H. M., P. M. Bennett, D. A. Lewis, and D. S. Reeves.** 1988. Cloning and characterization of an AAC(6') gene from *Serratia marcescens*. J Antimicrob Chemother **22:**587-96.
147. **Chan, J., X. D. Fan, S. W. Hunter, P. J. Brennan, and B. R. Bloom.** 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect Immun **59:**1755-61.
148. **Chan, P. F., M. Lonetto, S. M. Clark, R. Gagnon, L. Palmer, G. Woodnutt, P. Warren, and D. D. Jaworski.** 2001. Global expression analysis for the study of mode of action in *Staphylococcus aureus*. ASM conference **Poster A-26:** Florida, USA.
149. **Chang, M. J., and L. L. Barton.** 1974. *Mycobacterium fortuitum* osteomyelitis of the calcaneus secondary to a puncture wound. J Pediatr **85:**517-9.
150. **Chaplin, C. E.** 1952. Bacterial resistance to quaternary ammonium disinfectants. J Bacteriol **63:**453-8.
151. **Chapman, J. S.** 2003. Biocide resistance mechanisms. Int Biodeterior Biodegradation **51:**133-138.
152. **Chapman, J. S.** 1998. Characterizing bacterial resistance to preservatives and disinfectants. Int Biodeterior Biodegradation **41:**241–245.
153. **Chargaff, E., M. C. Pangborn, and R. J. Anderson.** 1931. The chemistry of the lipoids of tubercle bacilli. XXIII. Separation of the lipoid fractions from the Timothy bacillus. J Biol Chem **90:**45-55.
154. **Chatterjee, D., S. W. Hunter, M. McNeil, and P. J. Brennan.** 1992. Lipoarabinomannan. Multiglycosylated form of the mycobacterial mannosylphosphatidylinositols. J Biol Chem **267:**6228-33.
155. **Chen, C. J., D. M. Tobiason, C. E. Thomas, W. M. Shafer, H. S. Seifert, and P. F. Sparling.** 2004. A mutant form of the *Neisseria gonorrhoeae* pilus secretin protein PilQ allows increased entry of heme and antimicrobial compounds. J Bacteriol **186:**730-9.
156. **Chen, J., T. Kuroda, M. N. Huda, T. Mizushima, and T. Tsuchiya.** 2003. An RND-type multidrug efflux pump SdeXY from *Serratia marcescens*. J Antimicrob Chemother **52:**176-9.
157. **Chen, J., E. W. Lee, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2003. Multi drug resistance in *Serratia marcescens* and cloning of genes responsible for the resistance. Biol Pharm Bull **26:**391-3.
158. **Chen, J., Y. Morita, M. N. Huda, T. Kuroda, T. Mizushima, and T. Tsuchiya.** 2002. VmrA, a member of a novel class of Na(+)-coupled multidrug efflux pumps from *Vibrio parahaemolyticus*. J Bacteriol **184:**572-6.

159. **Chen, R., W. Schmidmayr, C. Kramer, U. Chen-Schmeisser, and U. Henning.** 1980. Primary structure of major outer membrane protein II (OmpA protein) of *Escherichia coli* K-12. Proc Natl Acad Sci USA 77:4592-6.
160. **Chetchotisakd, P., P. Mootsikapun, S. Anunnatsiri, K. Jirarattanapochai, C. Choonhakarn, A. Chaiprasert, P. N. Ubol, L. J. Wheat, and T. E. Davis.** 2000. Disseminated infection due to rapidly growing mycobacteria in immunocompetent hosts presenting with chronic lymphadenopathy: a previously unrecognized clinical entity. Clin Infect Dis **30**:29-34.
161. **Chopra, I.** 1991. Bacterial resistance to disinfectants, antiseptics and toxic metal ions, p. 45-65. *In* S. P. Denyer and W. B. Hugo (ed.), Mechanisms of action of chemical biocides: their study and exploitation. Blackwell Scientific Publications, London, England.
162. **Chopra, I.** 1987. Microbial resistance to veterinary disinfectants and antiseptics, p. 43–65. *In* A. H. Linton, W. B. Hugo and A. D. Russell (ed.), Disinfection in veterinary and farm animal practice. Blackwell Scientific Publications, Oxford, England.
163. **Chow, J. W., and D. M. Shlaes.** 1991. Imipenem resistance associated with the loss of a 40 kDa outer membrane protein in *Enterobacter aerogenes*. J Antimicrob Chemother **28**:499-504.
164. **Christensen, A. B., K. Riedel, L. Eberl, L. R. Flodgaard, S. Molin, L. Gram, and M. Givskov.** 2003. Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. Microbiology **149**:471-83.
165. **Christensen, B. B., C. Sternberg, J. B. Andersen, L. Eberl, S. Moller, M. Givskov, and S. Molin.** 1998. Establishment of new genetic traits in a microbial biofilm community. Appl Environ Microbiol **64**:2247-55.
166. **Chuanchuen, R., K. Beinlich, T. T. Hoang, A. Becher, R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. Antimicrob Agents Chemother **45**:428-32.
167. **Chuanchuen, R., R. R. Karkhoff-Schweizer, and H. P. Schweizer.** 2003. High-level triclosan resistance in *Pseudomonas aeruginosa* is solely a result of efflux. Am J Infect Control **31**:124-7.
168. **Chuanchuen, R., C. T. Narasaki, and H. P. Schweizer.** 2002. The MexJK efflux pump of *Pseudomonas aeruginosa* requires OprM for antibiotic efflux but not for efflux of triclosan. J Bacteriol **184**:5036-44.
169. **Chung, Y. J., and M. H. Saier, Jr.** 2002. Overexpression of the *Escherichia coli sugE* gene confers resistance to a narrow range of quaternary ammonium compounds. J Bacteriol **184**:2543-5.
170. **Chung, Y. J., and M. H. Saier, Jr.** 2001. SMR-type multidrug resistance pumps. Curr Opin Drug Discov Devel **4**:237-45.
171. **Ciak, J., and F. E. Hahn.** 1967. Quinacrine (atebrin): mode of action. Science **156**:655-6.
172. **Cifone, M. A., and I. J. Fidler.** 1981. Increasing metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms. Proc Natl Acad Sci USA **78**:6949-52.
173. **Clark, R. B.** 1996. Imipenem resistance among *Acinetobacter baumannii*: association with reduced expression of a 33-36 kDa outer membrane protein. J Antimicrob Chemother **38**:245-51.
174. **Clarke, A. R.** 1996. Molecular chaperones in protein folding and translocation. Curr Opin Struct Biol **6**:43-50.
175. **Clarke, B. R., L. Cuthbertson, and C. Whitfield.** 2004. Nonreducing terminal modifications determine the chain length of polymannose O antigens of *Escherichia coli* and couple chain termination to polymer export via an ATP-binding cassette transporter. J Biol Chem **279**:35709-18.
176. **Clegg, H. W., M. T. Foster, W. E. Sanders, Jr., and W. B. Baine.** 1983. Infection due to organisms of the *Mycobacterium fortuitum* complex after augmentation mammaplasty: clinical and epidemiologic features. J Infect Dis **147**:427-33.
177. **Coates, D., and J. E. Death.** 1978. Sporicidal activity of mixtures of alcohol and hypochlorite. J Clin Pathol **31**:148-52.
178. **Coates, D., and D. N. Hutchinson.** 1994. How to produce a hospital disinfection policy. J Hosp Infect **26**:57-68.

179. **Codling, C. E., B. V. Jones, E. Mahenthiralingam, A. D. Russell, and J. Y. Maillard.** 2004. Identification of genes involved in the susceptibility of *Serratia marcescens* to polyquaternium-1. J Antimicrob Chemother **54:**370-5.
180. **Codling, C. E., J. Y. Maillard, and A. D. Russell.** 2003. Aspects of the antimicrobial mechanisms of action of a polyquaternium and an amidoamine. J Antimicrob Chemother **51:**1153-8.
181. **Coia, J. E., G. J. Duckworth, D. I. Edwards, M. Farrington, C. Fry, H. Humphreys, C. Mallaghan, and D. R. Tucker.** 2006. Guidelines for the control and prevention of meticillin-resistant *Staphylococcus aureus* (MRSA) in healthcare facilities. J Hosp Infect **63:**1-44.
182. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, and *et al.*** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-44.
183. **Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, and *et al.*** 2001. Massive gene decay in the leprosy bacillus. Nature **409:**1007-11.
184. **Coleman, D. C., H. Pomeroy, J. K. Estridge, C. T. Keane, M. T. Cafferkey, R. Hone, and T. J. Foster.** 1985. Susceptibility to antimicrobial agents and analysis of plasmids in gentamicin- and methicillin-resistant *Staphylococcus aureus* from Dublin hospitals. J Med Microbiol **20:**157-67.
185. **Collier, P. J., A. J. Ramsey, P. Austin, and P. Gilbert.** 1990. Growth inhibitory and biocidal activity of some isothiazolone biocides. J Appl Bacteriol **69:**569-77.
186. **Collins, F. M.** 1986. Bactericidal activity of alkaline glutaraldehyde solution against a number of atypical mycobacterial species. J Appl Bacteriol **61:**247-51.
187. **Colwell, R. R., and M. Mandel.** 1965. Adansonian analysis and deoxyribonucleic acid base composition of *Serratia marcescens*. J Bacteriol **89:**454-61.
188. **Condon, C., R. Cammack, D. S. Patil, and P. Owen.** 1985. The succinate dehydrogenase of *Escherichia coli*. Immunochemical resolution and biophysical characterization of a 4-subunit enzyme complex. J Biol Chem **260:**9427-34.
189. **Cookson, B. D., M. C. Bolton, and J. H. Platt.** 1991. Chlorhexidine resistance in methicillin-resistant *Staphylococcus aureus* or just an elevated MIC? An in vitro and in vivo assessment. Antimicrob Agents Chemother **35:**1997-2002.
190. **Cookson, B. D., H. Farrelly, P. Stapleton, R. P. Garvey, and M. R. Price.** 1991. Transferable resistance to triclosan in MRSA. Lancet **337:**1548-9.
191. **Cooper, C. L., M. Wiseman, and R. Brunham.** 1998. Bullous cellulitis caused by *Serratia marcescens*. Int J Infect Dis **3:**36-8.
192. **Coria-Jimenez, R., and C. Ortiz-Torres.** 1994. Aminoglycoside resistance patterns of *Serratia marcescens* strains of clinical origin. Epidemiol Infect **112:**125-31.
193. **Cornelis, G. R., C. Sluiters, I. Delor, D. Geib, K. Kaniga, C. Lambert de Rouvroit, M. P. Sory, J. C. Vanooteghem, and T. Michiels.** 1991. *ymoA*, a *Yersinia enterocolitica* chromosomal gene modulating the expression of virulence functions. Mol Microbiol **5:**1023-34.
194. **Corrales, F. J., and A. R. Fersht.** 1996. Toward a mechanism for GroEL.GroES chaperone activity: an ATPase-gated and -pulsed folding and annealing cage. Proc Natl Acad Sci USA **93:**4509-12.
195. **Cottone, J. A., and J. A. Molinari.** 1996. Disinfectant use in dentistry, p. 73–82. *In* J. M. Ascenzi, Handbook of disinfectants and antiseptics. Marcel Dekker, New York.
196. **Coulthurst, S. J., N. R. Williamson, A. K. Harris, D. R. Spring, and G. P. Salmond.** 2006. Metabolic and regulatory engineering of *Serratia marcescens*: mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum-sensing capacities. Microbiology **152:**1899-911.
197. **Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma, Jr.** 1999. Occurrence of nontuberculous mycobacteria in environmental samples. Appl Environ Microbiol **65:**2492-6.
198. **Cox, A. D., and S. G. Wilkinson.** 1991. Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance. Mol Microbiol **5:**641-6.
199. **Cozens, R. M., E. Tuomanen, W. Tosch, O. Zak, J. Suter, and A. Tomasz.** 1986. Evaluation of the bactericidal activity of beta-lactam antibiotics on slowly growing bacteria cultured in the chemostat. Antimicrob Agents Chemother **29:**797-802.

200. **Crosa, J. H., and C. T. Walsh.** 2002. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev **66:**223-49.
201. **Croshaw, B.** 1971. The destruction of mycobacteria, p. 420–449. *In* W. B. Hugo (ed.), Inhibition and destruction of the microbial cell. Academic Press, London, England.
202. **Cudic, M., B. A. Condie, D. J. Weiner, E. S. Lysenko, Z. Q. Xiang, O. Insug, P. Bulet, and L. Otvos, Jr.** 2002. Development of novel antibacterial peptides that kill resistant isolates. Peptides **23:**2071-83.
203. **Cullen, A. R., C. L. Cannon, E. J. Mark, and A. A. Colin.** 2000. *Mycobacterium abscessus* infection in cystic fibrosis. Colonization or infection? Am J Respir Crit Care Med **161:**641-5.
204. **Cunha, C. D., M. do Rosario, A. S. Rosado, and S. G. F. Leite.** 2004. *Serratia* sp. SVGG16: a promising biosurfactant producer isolated from tropical soil during growth with ethanol blended gasoline. Process Biochem **39:**2277–82.
205. **Cutay, A. M., H. W. Horowitz, R. W. Pooley, K. Van Horn, and G. P. Wormser.** 1998. Infection of epicardial pacemaker wires due to *Mycobacterium abscessus*. Clin Infect Dis **26:**520-1.
206. **Cuthbertson, L., J. Powers, and C. Whitfield.** 2005. The C-terminal domain of the nucleotide-binding domain protein Wzt determines substrate specificity in the ATP-binding cassette transporter for the lipopolysaccharide O-antigens in *Escherichia coli* serotypes O8 and O9a. J Biol Chem **280:**30310-9.
207. **Cutler, R. R., and P. Wilson.** 1993. Disinfectant testing of contaminated endoscopes--a need for standardization. J Hosp Infect **25:**145-7.
208. **D'Arcy, P. F., and E. P. Taylor.** 1962. Quaternary ammonium compounds in medicinal chemistry. I. J Pharm Pharmacol **14:**129-46.
209. **Daffe, M., P. J. Brennan, and M. McNeil.** 1990. Predominant structural features of the cell wall arabinogalactan of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycosyl alditol fragments by gas chromatography/mass spectrometry and by 1H and 13C NMR analyses. J Biol Chem **265:**6734-43.
210. **Dai, K., and J. Lutkenhaus.** 1991. *ftsZ* is an essential cell division gene in *Escherichia coli.* J Bacteriol **173:**3500-6.
211. **Daldal, F., and D. G. Fraenkel.** 1981. *Tn*10 insertions in the *pfkB* region of *Escherichia coli.* J Bacteriol **147:**935-43.
212. **Dame, R. T.** 2005. The role of nucleoid-associated proteins in the organization and compaction of bacterial chromatin. Mol Microbiol **56:**858-70.
213. **Dandanell, G., K. Norris, and K. Hammer.** 1991. Long-distance *deoR* regulation of gene expression in *Escherichia coli.* Ann N Y Acad Sci **646:**19-30.
214. **Danese, P. N., and T. J. Silhavy.** 1998. CpxP, a stress-combative member of the Cpx regulon. J Bacteriol **180:**831-9.
215. **Daniels, C., C. Vindurampulle, and R. Morona.** 1998. Overexpression and topology of the *Shigella flexneri* O-antigen polymerase (Rfc/Wzy). Mol Microbiol **28:**1211-22.
216. **David, H. L., N. Rastogi, S. Clavel-Seres, F. Clement, and M. F. Thorel.** 1987. Structure of the cell envelope of *Mycobacterium avium*. Zentralbl Bakteriol Mikrobiol Hyg **264:**49-66.
217. **Davies, A., M. Bentley, and B. S. Field.** 1968. Comparison of the action of vantocil, cetrimide and chlorhexidine on *Escherichia coli* and its spheroplasts and the protoplasts of gram positive bacteria. J Appl Bacteriol **31:**448-61.
218. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295-8.
219. **Davies, G. E., J. Francis, A. R. Martin, F. L. Rose, and G. Swain.** 1954. 1:6-Di-4'-chlorophenyldiguanidohexane (hibitane); laboratory investigation of a new antibacterial agent of high potency. Br J Pharmacol Chemother **9:**192-6.
220. **Davis, B. R., W. H. Ewing, and R. V. Reavis.** 1957. The biochemical reactions given by members of the *Serratia* group. Int Bull Bacteriol Nomencl Taxon **7:**152-60
221. **Davis, J. G.** 1962. Idophors as detergent-sterilizers. J Appl Bacteriol **25:**195–201.
222. **De La Rosa, E. J., M. A. De Pedro, and D. Vasquez.** 1982. Modification of penicillin binding proteins of *Escherichia coli* associated with changes in the state of growth of the cells. FEMS Microbiol Lett **14:**91-4.

223. **De Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J Bacteriol **172:**6568-72.
224. **De Maat, C. E., A. M. Kayser, and J. C. van der Wal.** 1974. *Serratia marcescens* septicemia in a previously healthy woman. Ned Tijdschr Geneeskd **118:**502-5.
225. **De Rossi, E., J. A. Ainsa, and G. Riccardi.** 2006. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS Microbiol Rev **30:**36-52.
226. **Dechend, R., F. Hirano, K. Lehmann, V. Heissmeyer, S. Ansieau, F. G. Wulczyn, C. Scheidereit, and A. Leutz.** 1999. The Bcl-3 oncoprotein acts as a bridging factor between NF-kappaB/Rel and nuclear co-regulators. Oncogene **18:**3316-23.
227. **Del Sal, G., G. Manfioletti, and C. Schneider.** 1989. The CTAB-DNA precipitation method: a common mini-scale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. Biotechniques **7:**514-20.
228. **Delboni, L. F., S. C. Mande, F. Rentier-Delrue, V. Mainfroid, S. Turley, F. M. Vellieux, J. A. Martial, and W. G. Hol.** 1995. Crystal structure of recombinant triosephosphate isomerase from *Bacillus stearothermophilus*. An analysis of potential thermostability factors in six isomerases with known three-dimensional structures points to the importance of hydrophobic interactions. Protein Sci **4:**2594-604.
229. **Demain, A. L.** 1995. Why do microorganisms produce antimicrobials?, p. 205-28. *In* P. A. Hunter, G. K. Darby and N. J. Russel (ed.), Fifty years of antimicrobials: Past perspectives and future trends. Society for General Microbiology, Cambridge.
230. **Denton, G. W.** 1991. Chlorhexidine, p. 322-33. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation. Lea & Feabiger, Philadelphia.
231. **Denton, G. W.** 2001. Disinfectation, sterilization, and preservation 5th ed. Lippincott, Williams & Wilkins.
232. **Denyer, S.** 1995. Mechanisms of action of antibacterial biocides. Int Biodeterior Biodegrad **36:**227–45.
233. **Denyer, S. P., and J. Y. Maillard.** 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. J Appl Microbiol **92 (Suppl):** 35S-45S.
234. **Derzelle, S., E. Duchaud, F. Kunst, A. Danchin, and P. Bertin.** 2002. Identification, characterization, and regulation of a cluster of genes involved in carbapenem biosynthesis in *Photorhabdus luminescens*. Appl Environ Microbiol **68:**3780-9.
235. **Dessen, A., A. Quemard, J. S. Blanchard, W. R. Jacobs, Jr., and J. C. Sacchettini.** 1995. Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. Science **267:**1638-41.
236. **Dhandayuthapani, S., Y. Zhang, M. H. Mudd, and V. Deretic.** 1996. Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: characterization and inducibility of *ahpC* by peroxides in *Mycobacterium smegmatis* and lack of expression in *M. aurum* and *M. tuberculosis*. J Bacteriol **178:**3641-9.
237. **Dixon, B.** 2000. Antibiotics as growth promoters: risks and alternatives. ASM News **66:**264-265.
238. **Dodson, K. W., F. Jacob-Dubuisson, R. T. Striker, and S. J. Hultgren.** 1993. Outer-membrane PapC molecular usher discriminately recognizes periplasmic chaperone-pilus subunit complexes. Proc Natl Acad Sci USA **90:**3670-4.
239. **Doi, Y., K. Yokoyama, K. Yamane, J. Wachino, N. Shibata, T. Yagi, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrob Agents Chemother **48:**491-6.
240. **Donachie, W. D., and K. J. Begg.** 1970. Growth of the bacterial cell. Nature **227:**1220-4.
241. **Donzis, P. B., B. J. Mondino, B. A. Weissman, and D. A. Bruckner.** 1987. Microbial contamination of contact lens care systems. Am J Ophthalmol **104:**325-33.
242. **Doran, J. L., Y. Pang, K. E. Mdluli, A. J. Moran, T. C. Victor, R. W. Stokes, E. Mahenthiralingam, B. N. Kreiswirth, J. L. Butt, G. S. Baron, and *et al.*** 1997. *Mycobacterium tuberculosis efpA* encodes an efflux protein of the QacA transporter family. Clin Diagn Lab Immunol **4:**23-32.
243. **Dorman, C. J.** 2004. H-NS: a universal regulator for a dynamic genome. Nat Rev Microbiol **2:**391-400.

244. **Dorman, C. J., and P. Deighan.** 2003. Regulation of gene expression by histone-like proteins in bacteria. Curr Opin Genet Dev **13:**179-84.
245. **Dorrer, E., and M. Teuber.** 1977. Induction of polymyxin resistance in *Pseudomonas fluorescens* by phosphate limitation. Arch Microbiol **114:**87-9.
246. **Dorsey, C. W., and L. A. Actis.** 2004. Analysis of pVU3695, a plasmid encoding glutathione-dependent formaldehyde dehydrogenase activity and formaldehyde resistance in the *Escherichia coli* VU3695 clinical strain. Plasmid **51:**116-26.
247. **Drake, S. L., and M. Koomey.** 1995. The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. Mol Microbiol **18:**975-86.
248. **Draper, P.** 1998. The outer parts of the mycobacterial envelope as permeability barriers. Front Biosci **3:**D1253-61.
249. **Drechsel, H., and G. Jung.** 1998. Peptide siderophores. J Pept Sci **4:**147-81.
250. **Drlica, K., and J. Rouviere-Yaniv.** 1987. Histonelike proteins of bacteria. Microbiol Rev **51:**301-19.
251. **Dubouix, A., C. Roques, C. Segonds, M. J. Jeannot, S. Malavaud, S. Daude, G. Chabanon, and N. Marty.** 2005. Epidemiological investigation of a *Serratia liquefaciens* outbreak in a neurosurgery department. J Hosp Infect **60:**8-13.
252. **Dussurget, O., M. Rodriguez, and I. Smith.** 1996. An *ideR* mutant of *Mycobacterium smegmatis* has derepressed siderophore production and an altered oxidative-stress response. Mol Microbiol **22:**535-44.
253. **Eager, R. G., J. Leder, and A. B. Theis.** 1986. Glutaraldehyde: factors important for micobacterial efficacy. The 3rd Conference on Progress in Chemical Disinfection. SUNY, Binghamton, New York.
254. **Eberhard, A., C. A. Widrig, P. McBath, and J. B. Schineller.** 1986. Analogs of the autoinducer of bioluminescence in *Vibrio fischeri*. Arch Microbiol **146:**35-40.
255. **Eberl, L., S. Molin, and M. Givskov.** 1999. Surface motility of *Serratia liquefaciens* MG1. J Bacteriol **181:**1703-12.
256. **Eberl, L., M. K. Winson, C. Sternberg, G. S. Stewart, G. Christiansen, S. R. Chhabra, B. Bycroft, P. Williams, S. Molin, and M. Givskov.** 1996. Involvement of *N*-acyl-L-hormoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. Mol Microbiol **20:**127-36.
257. **Ehrenberg, C. G.** 1848. Monatsber. Berlin Konigl Pruess Akad Wiss:349-62.
258. **Ehrenkranz, N. J., E. A. Bolyard, M. Wiener, and T. J. Cleary.** 1980. Antibiotic-sensitive *Serratia marcescens* infections complicating cardiopulmonary operations: contaminated disinfectant as a reservoir. Lancet **2:**1289-92.
259. **Eisenberg, J.** 1886. Bakteriokgische Diagnostik. Hulfs- Tabellen beim Praktischen Arbeiten. Leipzig: Voss.
260. **Eklund, T.** 1985. The effect of sorbic acid and esters of p-hydroxybenzoic acid on the protonmotive force in *Escherichia coli* membrane vesicles. J Gen Microbiol **131:**73-6.
261. **Eklund, T.** 1985. Inhibition of microbial growth at different pH levels by benzoic and propionic acids and esters of p-hydroxybenzoic acid. Int J Food Microbiol **2:**159–167.
262. **El-Falaha, B. M. A., D. T. Rogers, A. D. Russell, and J. R. Furr.** 1985. Effect of some antibacterial agents on the hydrophobicity of wild type and envelope mutant of *Escherichia coli*. Curr Microbiol **12:**187–90.
263. **El-Falaha, B. M. A., A. D. Russell, and J. R. Furr.** 1985. Effects of chlorhexidine diacetate and benzalkonium chloride on the viability of wild type and envelope mutant of *Escherichia coli* and *Pseudomonas aeruginosa*. Lett Appl Microbiol **1:**21–24.
264. **Elferink, J. G., and H. L. Booij.** 1974. Interaction of chlorhexidine with yeast cells. Biochem Pharmacol **23:**1413-9.
265. **Elgrably-Weiss, M., E. Schlosser-Silverman, I. Rosenshine, and S. Altuvia.** 2006. DeoT, a DeoR-type transcriptional regulator of multiple target genes. FEMS Microbiol Lett **254:**141-8.
266. **ElHwood, D. C., and D. W. Tempest.** 1972. Effects of environment on bacterial wall content and composition. Adv Microb Physiol **7:**83-117.
267. **Ellis, R. J., and F. U. Hartl.** 1996. Protein folding in the cell: competing models of chaperonin function. Faseb J **10:**20-6.

268. **Ellison, D. W., B. Young, K. Nelson, and V. L. Miller.** 2003. YmoA negatively regulates expression of invasin from *Yersinia enterocolitica*. J Bacteriol **185:**7153-9.
269. **Ellison, R. T., 3rd, T. J. Giehl, and F. M. LaForce.** 1988. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. Infect Immun **56:**2774-81.
270. **Emanuel, B., M. Goldin, and M. Brauer.** 1963. *Serratia Marcescens* septicemia following artificial kidney dialysis. J Urol **90:**34-6.
271. **Engelhart, S., F. Saborowski, M. Krakau, G. Scherholz-Schlosser, I. Heyer, and M. Exner.** 2003. Severe *Serratia liquefaciens* sepsis following vitamin C infusion treatment by a naturopathic practitioner. J Clin Microbiol **41:**3986-8.
272. **Erridge, C., E. Bennett-Guerrero, and I. R. Poxton.** 2002. Structure and function of lipopolysaccharides. Microbes Infect **4:**837-51.
273. **Esther, C. R., Jr., M. M. Henry, P. L. Molina, and M. W. Leigh.** 2005. Nontuberculous mycobacterial infection in young children with cystic fibrosis. Pediatr Pulmonol **40:**39-44.
274. **Etienne, G., F. Laval, C. Villeneuve, P. Dinadayala, A. Abouwarda, D. Zerbib, A. Galamba, and M. Daffe.** 2005. The cell envelope structure and properties of *Mycobacterium smegmatis* mc(2)155: is there a clue for the unique transformability of the strain? Microbiology **151:**2075-86.
275. **Ewing, W. H., B. R. Davis, and J. G. Johnson.** 1962. Int Bull Bacteriol Nomencl Taxon **12:**47-52.
276. **Fahlen, T. F., R. L. Wilson, J. D. Boddicker, and B. D. Jones.** 2001. Hha is a negative modulator of transcription of *hilA*, the *Salmonella enterica* serovar *Typhimurium* invasion gene transcriptional activator. J Bacteriol **183:**6620-9.
277. **Falcone, V., E. B. Bassey, A. Toniolo, P. G. Conaldi, and F. M. Collins.** 1994. Differential release of tumor necrosis factor-alpha from murine peritoneal macrophages stimulated with virulent and avirulent species of mycobacteria. FEMS Immunol Med Microbiol **8:**225-32.
278. **Falkinham, J. O., 3rd.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin Microbiol Rev **9:**177-215.
279. **Falkow, S., J. Marmur, W. F. Carey, W. M. Spilman, and L. S. Baron.** 1961. Episomic transfer between *Salmonella typhosa* and *Serratia marcescens*. Genetics **46:**703-6.
280. **Fan, F., K. Yan, N. G. Wallis, S. Reed, T. D. Moore, S. F. Rittenhouse, W. E. DeWolf, Jr., J. Huang, D. McDevitt, W. H. Miller, and *et al.*** 2002. Defining and combating the mechanisms of triclosan resistance in clinical isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother **46:**3343-7.
281. **Fang, C. T., H. C. Chen, Y. P. Chuang, S. C. Chang, and J. T. Wang.** 2002. Cloning of a cation efflux pump gene associated with chlorhexidine resistance in *Klebsiella pneumoniae*. Antimicrob Agents Chemother **46:**2024-8.
282. **Fass, R. J.** 1983. In vitro activity of ciprofloxacin (Bay o 9867). Antimicrob Agents Chemother **24:**568-74.
283. **Favero, M. S.** 1991. Practical application of liquid sterilants in health care facilities, p. 397-405. *In* R. F. Morrissey and Y. I. Prokopenko (ed.), Sterilization of medical products, vol. V. Polyscience Publications Morin Heights, Canada.
284. **Favero, M. S., and W. W. Bond.** 1991. Chemical disinfection of medical surgical material, p. 617–41, Disinfection, sterilization, and preservation. Lea & Febiger, Philadelphia.
285. **Favero, M. S., and W. W. Bond.** 1991. Chemical disinfection of medical and surgical materials, p. 617-41. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation, vol. 4. Lea & Febiger, Philadelphia.
286. **Favero, M. S., and W. W. Bond.** 1991. Sterilization, disinfection, and antisepsis in the hospital, p. 183-200. *In* A. Balows, W. J. Hausler, K. L. Herrmann, H. D. Isenberg and H. J. Shadomy (ed.), Manual of clinical microbiology, vol. 5. ASM, Washington, DC.
287. **Fayot-Petitmaire, M., J. Simon, and L. Hidden.** 1963. Post-abortion septicemia due to *Serratia marcescens*. Gaz Med Fr **70:**675-80.
288. **Feinman, S. E.** 1999. Antibiotics in animal feeds —drug resistance revisited. ASM News **64:**24-29.
289. **Ferng, S. F.** 2002. Ozone-induced DNA single strand-breaks in guinea pig tracheobronchial epithelial cells in vivo. Inhal Toxicol **14:**621-33.

290. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci USA **76:**1648-52.
291. **Finch, J. E., and M. R. Brown.** 1975. The influence of nutrient limitation in a chemostat on the sensitivity of *Pseudomonas aeruginosa* to polymyxin and to EDTA. J Antimicrob Chemother **1:**379-86.
292. **Finch, R. G., D. I. Pritchard, B. W. Bycroft, P. Williams, and G. S. Stewart.** 1998. Quorum sensing: a novel target for anti-infective therapy. J Antimicrob Chemother **42:**569-71.
293. **Finken, M., P. Kirschner, A. Meier, A. Wrede, and E. C. Bottger.** 1993. Molecular basis of streptomycin resistance in *Mycobacterium tuberculosis*: alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. Mol Microbiol **9:**1239-46.
294. **Fitzgerald, K. A., A. Davies, and A. D. Russell.** 1992. Mechanism of action of chlorhexidine diacetate and phenoxyethanol singly and in combination against gram-negative bacteria. Microbios **70:**215-30.
295. **Fitzgerald, K. A., A. Davies, and A. D. Russell.** 1989. Uptake of 14C-chlorhexidine diacetate to *Escherichia coli* and *Pseudomonas aeruginosa* and its release by azolectin. FEMS Microbiol Lett **51:**327-32.
296. **Fitzgerald, K. A., A. Davies, and A. D. Russell.** 1992. Effect of chlorhexidine and phenoxyethanol on cell surface hydrophobicity of Gram-positive and Gram-negative bacteria. Lett Appl Microbiol **14:**91–95.
297. **Flavier, A. B., S. J. Clough, M. A. Schell, and T. P. Denny.** 1997. Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. Mol Microbiol **26:**251-9.
298. **Flyg, C., K. Kenne, and H. G. Boman.** 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to *Cecropia* immunity and a decreased virulence to *Drosophila*. J Gen Microbiol **120:**173-81.
299. **Fodor, T., and I. Szabo.** 1980. Effect of chlorhexidine gluconate on the survival of acid fast bacteria. Acta Microbiol Acad Sci Hung **27:**343-4.
300. **Fortineau, L.** 1904. Erythrobacilluspyo-septicus et bacteries rouges. Fac. Midecine, Paris.
301. **Foulds, J., and C. Barrett.** 1973. Characterization of *Escherichia coli* mutants tolerant to bacteriocin JF246: two new classes of tolerant mutants. J Bacteriol **116:**885-92.
302. **Fox, L. E., G. A. Kunkle, B. L. Homer, C. Manella, and J. P. Thompson.** 1995. Disseminated subcutaneous *Mycobacterium fortuitum* infection in a dog. J Am Vet Med Assoc **206:**53-5.
303. **Fox, L. P., A. S. Geyer, S. Husain, P. Della-Latta, and M. E. Grossman.** 2004. *Mycobacterium abscessus* cellulitis and multifocal abscesses of the breasts in a transsexual from illicit intramammary injections of silicone. J Am Acad Dermatol **50:**450-4.
304. **Fraise, A. P.** 2002. Biocide abuse and antimicrobial resistance--a cause for concern? J Antimicrob Chemother **49:**11-2.
305. **Fraise, A. P.** 1999. Choosing disinfectants. J Hosp Infect **43:**255-64.
306. **Franco, A. V., D. Liu, and P. R. Reeves.** 1996. A Wzz (Cld) protein determines the chain length of K lipopolysaccharide in *Escherichia coli* O8 and O9 strains. J Bacteriol **178:**1903-7.
307. **Franco, A. V., D. Liu, and P. R. Reeves.** 1998. The *wzz* (*cld*) protein in *Escherichia coli*: amino acid sequence variation determines O-antigen chain length specificity. J Bacteriol **180:**2670-5.
308. **Franklin, D. J., J. R. Starke, M. T. Brady, B. A. Brown, and R. J. Wallace, Jr.** 1994. Chronic otitis media after tympanostomy tube placement caused by *Mycobacterium abscessus*: a new clinical entity? Am J Otol **15:**313-20.
309. **Fraser, V., and R. J. J. Wallace.** 1996. Nontuberculous mycobacteria. *In* C. G. Mayhall (ed.), Hospital epidemiology and infection control. Williams & Wilkins, Baltimore, USA.
310. **Fraud, S., A. C. Hann, J. Y. Maillard, and A. D. Russell.** 2003. Effects of *ortho*-phthalaldehyde, glutaraldehyde and chlorhexidine diacetate on *Mycobacterium chelonae* and *Mycobacterium abscessus* strains with modified permeability. J Antimicrob Chemother **51:**575-84.
311. **Fraud, S., J. Y. Maillard, and A. D. Russell.** 2001. Comparison of the mycobactericidal activity of *ortho*- phthalaldehyde, glutaraldehyde and other dialdehydes by a quantitative suspension test. J Hosp Infect **48:**214-21.

312. **Frederick, J. J., T. R. Corner, and P. Gerhardt.** 1974. Antimicrobial actions of hexachlorophene: inhibition of respiration in *Bacillus megaterium*. Antimicrob Agents Chemother **6:**712-21.
313. **Freney, J., M. O. Husson, F. Gavini, S. Madier, A. Martra, D. Izard, H. Leclerc, and J. Fleurette.** 1988. Susceptibilities to antibiotics and antiseptics of new species of the family *Enterobacteriaceae*. Antimicrob Agents Chemother **32:**873-6.
314. **Frere, J. M.** 1989. Quantitative relationship between sensitivity to beta-lactam antibiotics and beta-lactamase production in gram-negative bacteria--I. Steady-state treatment. Biochem Pharmacol **38:**1415-26.
315. **Frere, J. M., B. Joris, M. Crine, and H. H. Martin.** 1989. Quantitative relationship between sensitivity to beta-lactam antibiotics and beta-lactamase production in gram-negative bacteria--II. Non-steady-state treatment and progress curves. Biochem Pharmacol **38:**1427-33.
316. **Freudl, R., and S. T. Cole.** 1983. Cloning and molecular characterization of the *ompA* gene from *Salmonella typhimurium*. Eur J Biochem **134:**497-502.
317. **Frobisher, M., Jr., and L. Sommermeyer.** 1953. A study of the effect of alcohols on tubercle bacilli and other bacteria in sputum. Am Rev Tuberc **68:**419-24.
318. **Fu, H., A. A. Belaaouaj, C. Dahlgren, and J. Bylund.** 2003. Outer membrane protein A deficient *Escherichia coli* activates neutrophils to produce superoxide and shows increased susceptibility to antibacterial peptides. Microbes Infect **5:**781-8.
319. **Fujimaki, K., T. Fujii, H. Aoyama, K. Sato, Y. Inoue, M. Inoue, and S. Mitsuhashi.** 1989. Quinolone resistance in clinical isolates of *Serratia marcescens*. Antimicrob Agents Chemother **33:**785-7.
320. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol **176:**269-75.
321. **Furr, J. R.** 1996. Sensitivity of protozoa to disinfection *B. Acanthamoeba* and contact lens solutions. *In* A. D. Russell, W. B. Hugo and G. A. J. Ayliffe (ed.), Principles and practice of disinfection, preservation and sterilization. 3rd ed. Blackwell Scientific Publications, Oxford, England.
322. **Galil, K., L. A. Miller, M. A. Yakrus, R. J. Wallace, Jr., D. G. Mosley, B. England, G. Huitt, M. M. McNeil, and B. A. Perkins.** 1999. Abscesses due to *Mycobacterium abscessus* linked to injection of unapproved alternative medication. Emerg Infect Dis **5:**681-7.
323. **Gandhi, P. A., A. D. Sawant, L. A. Wilson, and D. G. Ahearn.** 1993. Adaptation and growth of *Serratia marcescens* in contact lens disinfectant solutions containing chlorhexidine gluconate. Appl Environ Microbiol **59:**183-8.
324. **Gao, L. Y., R. Groger, J. S. Cox, S. M. Beverley, E. H. Lawson, and E. J. Brown.** 2003. Transposon mutagenesis of *Mycobacterium marinum* identifies a locus linking pigmentation and intracellular survival. Infect Immun **71:**922-9.
325. **Gaora, P. O.** 1998. Expression of genes in mycobacteria. Methods Mol Biol **101:**261-73.
326. **Garcia, D. C., G. M. Woloj, S. Pineiro, D. O. Sordelli, and S. Kaufman.** 1995. An 8-year study of resistance to amikacin in gram-negative bacilli isolates from patients with nosocomial infection at one hospital in Argentina. J Med Microbiol **42:**283-90.
327. **Gargallo-Viola, D.** 1989. Enzyme polymorphism, prodigiosin production, and plasmid fingerprints in clinical and naturally occurring isolates of *Serratia marcescens*. J Clin Microbiol **27:**860-8.
328. **Gaughran, E. R.** 1969. From superstition to science: the history of a bacterium. Trans NY Acad Sci **31:**3-24.
329. **Gavin, J., N. F. Button, I. A. Watson-Craik, and N. A. Logan.** 1996. Efficacy of standard disinfectant test methods for contact lens-care solutions. Int Biodeterior Biodegradation **36:**431–440.
330. **Gehr, T. W., and B. A. Walters.** 1994. Catheter-related *Mycobacterium chelonei* infection in a CAPD patient. Perit Dial Int **14:**278-9.
331. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. I. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc Natl Acad Sci USA **74:**4772-6.

332. **George, K. M., Y. Yuan, D. R. Sherman, and C. E. Barry, 3rd.** 1995. The biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. Identification and functional analysis of CMAS-2. J Biol Chem **270:**27292-8.
333. **Gershenfeld, L.** 1956. A new iodine dairy sanitizer. Am J Pharm Sci Support Public Health **128:**335-9.
334. **Gershenfeld, L., W. B. Flagg, and B. Witlin.** 1954. Iodine as a tuberculocidal agent. Mil Surg **114:**172-83.
335. **Ghuysen, J. M.** 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol Rev **32:**425-64.
336. **Giaconi, J., R. Pham, and C. N. Ta.** 2002. Bilateral *Mycobacterium abscessus* keratitis after laser in situ keratomileusis. J Cataract Refract Surg **28:**887-90.
337. **Gibert, I., S. Calero, and J. Barbe.** 1990. Measurement of in vivo expression of *nrdA* and *nrdB* genes of *Escherichia coli* by using *lacZ* gene fusions. Mol Gen Genet **220:**400-8.
338. **Gilbert, P., and M. R. Brown.** 1980. Cell wall-mediated changes in sensitivity of *Bacillus megaterium* to chlorhexidine and 2-phenoxyethanol, associated with growth rate and nutrient limitation. J Appl Bacteriol **48:**223-30.
339. **Gilbert, P., and M. R. Brown.** 1978. Effect of R-plasmid RP1 and nutrient depletion on the gross cellular composition of *Escherichia coli* and its resistance to some uncoupling phenols. J Bacteriol **133:**1062-5.
340. **Gilbert, P., P. R. Byron, and M. R. Brown.** 1981. Application of models for envelope growth to cell length distribution data for *Pseudomonas aeruginosa* at various specific growth rates. Microbios **31:**189-203.
341. **Gilbert, P., and A. J. McBain.** 2001. Biocide usage in the domestic setting and concern about antibacterial and antibiotic resistance. J Infect **43:**85-91.
342. **Gilbert, P., and A. J. McBain.** 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin Microbiol Rev **16:**189-208.
343. **Gilbert, P., D. Pemberton, and D. E. Wilkinson.** 1990. Barrier properties of the gram-negative cell envelope towards high molecular weight polyhexamethylene biguanides. J Appl Bacteriol **69:**585-92.
344. **Gilbert, P., and N. E. Wright.** 1986. Non-plasmidic resistance towards preservatives in pharmaceutical and cosmetic products. Soc Appl Bacteriol Tech Ser **22:**255-79.
345. **Gilby, A. R., and A. V. Few.** 1960. Lysis of protoplasts of *Micrococcus lysodeikticus* by ionic detergents. J Gen Microbiol **23:**19-26.
346. **Gillespie, M. T., B. R. Lyon, and R. A. Skurray.** 1989. Gentamicin and antiseptic resistance in epidemic methicillin-resistant *Staphylococcus aureus*. Lancet **1:**503.
347. **Gillespie, M. T., B. R. Lyon, and R. A. Skurray.** 1990. Typing of methicillin-resistant *Staphylococcus aureus* by antibiotic resistance phenotypes. J Med Microbiol **31:**57-64.
348. **Gillespie, M. T., and R. A. Skurray.** 1986. Plasmids in multiresistant *Staphylococcus aureus*. Microbiol Sci **3:**53-8.
349. **Gillis, M., J. De Ley, and M. De Cleene.** 1970. The determination of molecular weight of bacterial genome DNA from renaturation rates. Eur J Biochem **12:**143-53.
350. **Gilmour, M. W., N. R. Thomson, M. Sanders, J. Parkhill, and D. E. Taylor.** 2004. The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. Plasmid **52:**182-202.
351. **Gimeno, C., D. Navarro, F. Savall, E. Millas, M. A. Farga, J. Garau, R. Cisterna, and J. Garcia-de-Lomas.** 1996. Relationship between outer membrane protein profiles and resistance to ceftazidime, imipenem, and ciprofloxacin in *Pseudomonas aeruginosa* isolates from bacteremic patients. Eur J Clin Microbiol Infect Dis **15:**82-5.
352. **Gittelson, B. L., and I. O. Walker.** 1967. The interaction of proflavine with deoxyribonucleic acid and deoxyribonucleohistone. Biochim Biophys Acta **138:**619-21.
353. **Givskov, M., R. de Nys, M. Manefield, L. Gram, R. Maximilien, L. Eberl, S. Molin, P. D. Steinberg, and S. Kjelleberg.** 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. J Bacteriol **178:**6618-22.

354. **Godessart, N., F. J. Munoa, M. Regue, and A. Juarez.** 1988. Chromosomal mutations that increase the production of a plasmid-encoded haemolysin in *Escherichia coli*. J Gen Microbiol **134**:2779-87.
355. **Gordon, R. E., and M. M. Smith.** 1953. Rapidly growing, acid fast bacteria. I. Species' descriptions of *Mycobacterium phlei* Lehmann and Neumann and *Mycobacterium smegmatis* (Trevisan) Lehmann and Neumann. J Bacteriol **66**:41-8.
356. **Gorman, S. P., and E. M. Scott.** 1985. A comparative evaluation of dental aspirator cleansing and disinfectant solutions. Br Dent J **158**:13-6.
357. **Gorman, S. P., E. M. Scott, and E. P. Hutchinson.** 1984. Interaction of the *Bacillus subtilis* spore protoplast, cortex, ion-exchange and coatless forms with glutaraldehyde. J Appl Bacteriol **56**:95-102.
358. **Gorman, S. P., E. M. Scott, and A. D. Russell.** 1980. Antimicrobial activity, uses and mechanism of action of glutaraldehyde. J Appl Bacteriol **48**:161-90.
359. **Gottardi, W.** 2001. Iodine and iodine compounds, p. 159–83. *In* S. S. Block (ed.), Disinfection, sterilization and preservation, 5th ed. Lippincott Williams & Wilkins, Philadelphia., USA.
360. **Graham, L. L., T. J. Beveridge, and N. Nanninga.** 1991. Periplasmic space and the concept of the periplasm. Trends Biochem Sci **16**:328-9.
361. **Grebe, T., J. Paik, and R. Hakenbeck.** 1997. A novel resistance mechanism against beta-lactams in *Streptococcus pneumoniae* involves CpoA, a putative glycosyltransferase. J Bacteriol **179**:3342-9.
362. **Greene, A. K., B. K. Few, and J. C. Serafini.** 1993. A comparison of ozonation and chlorination for the disinfection of stainless steel surfaces. J Dairy Sci **76**:3617-20.
363. **Gregory, A. W., G. B. Schaalje, J. D. Smart, and R. A. Robison.** 1999. The mycobactericidal efficacy of *ortho*-phthalaldehyde and the comparative resistances of *Mycobacterium bovis*, *Mycobacterium terrae*, and *Mycobacterium chelonae*. Infect Control Hosp Epidemiol **20**:324-30.
364. **Gremillion, D. H., S. B. Mursch, and C. J. Lerner.** 1983. Injection site abscesses caused by *Mycobacterium chelonei*. Infect Control **4**:25-8.
365. **Griffith, D. E., W. M. Girard, and R. J. Wallace, Jr.** 1993. Clinical features of pulmonary disease caused by rapidly growing mycobacteria. An analysis of 154 patients. Am Rev Respir Dis **147**:1271-8.
366. **Griffiths, P. A., J. R. Babb, and A. P. Fraise.** 1999. Mycobactericidal activity of selected disinfectants using a quantitative suspension test. J Hosp Infect **41**:111-21.
367. **Griffiths, P. A., J. R. Babb, and A. P. Fraise.** 1998. *Mycobacterium terrae*: a potential surrogate for *Mycobacterium tuberculosis* in a standard disinfectant test. J Hosp Infect **38**:183-92.
368. **Grimont, F., and P. A. D. Grimont.** 1992. The genus *Serratia*, p. 2823-48. *In* A. Balowes, H. G. Truper, M. Dworkin, W. Harder and K. H. Schleifer (ed.), The prokaryotes – a handbook on the biology of bacteria: Ecophysiology, isolation, identification, application, vol. III. Springer Verlag, New York, NY.
369. **Grimont, P. A. D., and H. L. Dulong de Rosnay.** 1972. Numerical study of 60 strains of *Serratia*. J Gen Microbiol **72**:259-68.
370. **Grimont, P. A. D., and F. Grimont.** 1978. The genus *Serratia*. Annu Rev Microbiol **32**:221-48.
371. **Grimont, P. A. D., F. Grimont, and H. L. De Rosnay.** 1977. Taxonomy of the genus *Serratia*. J Gen Microbiol **98**:39-66.
372. **Grimont, P. A. D.** 1977. Le genre *Serratia*. Taxonomie et approche ecologique. Univ. Bordeaux I, Fiance.
373. **Grimont, P. A. D., and F. Grimont.** 1984. *Enterobacteriaceae*: Genus *Serratia*, p. 477-84. *In* N. L. Krieg and G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, London, UK.
374. **Grimont, P. A. D., F. Grimont, and H. L. C. Dulong.** 1964. List of Cultures, no. 7, p. 65. *In* N. C. o. P. P. Bacteria. (ed.), Taxonomic biochemistry and serology. Ministry of Agriculture, Fisheries, and Food, Harpenden, England.
375. **Grkovic, S., M. H. Brown, N. J. Roberts, I. T. Paulsen, and R. A. Skurray.** 1998. QacR is a repressor protein that regulates expression of the *Staphylococcus aureus* multidrug efflux pump QacA. J Biol Chem **273**:18665-73.

376. **Grohskopf, L. A., V. R. Roth, D. R. Feikin, M. J. Arduino, L. A. Carson, J. I. Tokars, S. C. Holt, B. J. Jensen, R. E. Hoffman, and W. R. Jarvis.** 2001. *Serratia liquefaciens* bloodstream infections from contamination of epoetin alfa at a hemodialysis center. N Engl J Med **344:**1491-7.
377. **Grooters, A. M., C. G. Couto, J. M. Andrews, S. E. Johnson, J. J. Kowalski, and R. B. Esplin.** 1995. Systemic *Mycobacterium smegmatis* infection in a dog. J Am Vet Med Assoc **206:**200-2.
378. **Grossgebauer, K.** 1970. Virus disinfection, p. 103–48. *In* M. Benarde (ed.), Disinfection. Marcel Dekker, New York.
379. **Grossman, N., M. A. Schmetz, J. Foulds, E. N. Klima, V. E. Jimenez-Lucho, L. L. Leive, and K. A. Joiner.** 1987. Lipopolysaccharide size and distribution determine serum resistance in *Salmonella montevideo*. J Bacteriol **169:**856-63.
380. **Grozdanov, L., U. Zahringer, G. Blum-Oehler, L. Brade, A. Henne, Y. A. Knirel, U. Schombel, J. Schulze, U. Sonnenborn, G. Gottschalk, and *et al.*** 2002. A single nucleotide exchange in the *wzy* gene is responsible for the semirough O6 lipopolysaccharide phenotype and serum sensitivity of *Escherichia coli* strain Nissle 1917. J Bacteriol **184:**5912-25.
381. **Guasch, J. F., S. Ferrer, J. Enfedaque, M. B. Viejo, and M. Regue.** 1995. A 17 kDa outer-membrane protein (Omp4) from *Serratia marcescens* confers partial resistance to bacteriocin 28b when expressed in *Escherichia coli*. Microbiology **141:**2535-42.
382. **Gueiros-Filho, F. J., and S. M. Beverley.** 1997. Trans-kingdom transposition of the *Drosophila* element mariner within the protozoan *Leishmania*. Science **276:**1716-9.
383. **Guerin-Mechin, L., F. Dubois-Brissonnet, B. Heyd, and J. Y. Leveau.** 2000. Quaternary ammonium compound stresses induce specific variations in fatty acid composition of *Pseudomonas aeruginosa*. Int J Food Microbiol **55:**157-9.
384. **Guerin-Mechin, L., F. Dubois-Brissonnet, B. Heyd, and J. Y. Leveau.** 1999. Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by quaternary ammonium compounds and relation with resistance to bactericidal activity. J Appl Microbiol **87:**735-42.
385. **Gupta, A., K. Matsui, J. F. Lo, and S. Silver.** 1999. Molecular basis for resistance to silver cations in *Salmonella*. Nat Med **5:**183-8.
386. **Gupta, A., and S. Silver.** 1998. Silver as a biocide: will resistance become a problem? Nat Biotechnol **16:**888.
387. **Gurley, B.** 1985. Ozone: pharmaceutical sterilant of the future? J Parenter Sci Technol **39:**256-61.
388. **Gutmann, L., R. Williamson, N. Moreau, M. D. Kitzis, E. Collatz, J. F. Acar, and F. W. Goldstein.** 1985. Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella, Enterobacter,* and *Serratia*. J Infect Dis **151:**501-7.
389. **Haefeli, C., C. Franklin, and K. Hardy.** 1984. Plasmid-determined silver resistance in *Pseudomonas stutzeri* isolated from a silver mine. J Bacteriol **158:**389-92.
390. **Haemers, A., D. C. Leysen, W. Bollaert, M. Q. Zhang, and S. R. Pattyn.** 1990. Influence of N substitution on antimycobacterial activity of ciprofloxacin. Antimicrob Agents Chemother **34:**496-7.
391. **Hagerhall, C.** 1997. Succinate: quinone oxidoreductases. Variations on a conserved theme. Biochim Biophys Acta **1320:**107-41.
392. **Hakim, R. M., R. A. Friedrich, and E. G. Lowrie.** 1985. Formaldehyde kinetics and bacteriology in dialyzers. Kidney Int **28:**936-43.
393. **Hallett, P., A. Mehlert, and A. Maxwell.** 1990. *Escherichia coli* cells resistant to the DNA gyrase inhibitor, ciprofloxacin, overproduce a 60 kD protein homologous to GroEL. Mol Microbiol **4:**345-53.
394. **Hamilton, W. A.** 1971. Membrane-active antibacterial compounds, p. 77–93. *In* W. B. Hugo (ed.), Inhibition and destruction of the microbial cell. Academic Press, London, England.
395. **Hamma, T., and A. R. Ferre-D'Amare.** 2006. Pseudouridine synthases. Chem Biol **13:**1125-35.
396. **Hammer-Jespersen, K.** 1983. Nucleoside catabolism, p. 203–58. *In* A. Munch-Petersen (ed.), Metabolism of nucleotides, nucleosides and nucleobasesin microorganisms. Academic Press, London, England.

397. **Hancock, R. E.** 1981. Aminoglycoside uptake and mode of action-with special reference to streptomycin and gentamicin. II. Effects of aminoglycosides on cells. J Antimicrob Chemother **8:**429-45.
398. **Hancock, R. E.** 1997. The bacterial outer membrane as a drug barrier. Trends Microbiol **5:**37-42.
399. **Hancock, R. E., and P. Reeves.** 1975. Bacteriophage resistance in *Escherichia coli* K-12: general pattern of resistance. J Bacteriol **121:**983-93.
400. **Harnett, S. J., K. D. Allen, and R. R. Macmillan.** 2001. Critical care unit outbreak of *Serratia liquefaciens* from contaminated pressure monitoring equipment. J Hosp Infect **47:**301-7.
401. **Harold, F. M., J. R. Baarda, C. Baron, and A. Abrams.** 1969. Inhibition of membrane-bound adenosine triphosphatase and of cation transport in *Streptococcus faecalis* by N,N'-dicyclohexylcarbodiimide. J Biol Chem **244:**2261-8.
402. **Harrison, F. C.** 1924. The miraculous microorganism. Trans R Soc Can **18:**1-17.
403. **Hartl, F. U.** 1996. Molecular chaperones in cellular protein folding. Nature **381:**571-9.
404. **Hashizume, T., M. Sanada, S. Nakagawa, and N. Tanaka.** 1993. Alteration in expression of *Serratia marcescens* porins associated with decreased outer membrane permeability. J Antimicrob Chemother **31:**21-8.
405. **Havarstein, L. S., G. Coomaraswamy, and D. A. Morrison.** 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc Natl Acad Sci USA **92:**11140-4.
406. **Hawkey, P. M.** 2004. Mycobactericidal agents. *In* P. A. Fraise, A. P. Lambert and J-Y. Maillard (ed.), Russell, Hugo & Ayliffe's Principles and practice of disinfection, preservation and sterilization, vol. 4. Blackwell Publishing, Oxford.
407. **Hawkey, P. M., and H. K. Constable.** 1988. Selection of netilmicin resistance, associated with increased 6' aminoglycoside acetyltransferase activity, in *Serratia marcescens*. J Antimicrob Chemother **21:**535-44.
408. **Hayes, P. S., D. L. McGiboney, J. D. Band, and J. C. Feeley.** 1982. Resistance of *Mycobacterium chelonei*-like organisms to formaldehyde. Appl Environ Microbiol **43:**722-4.
409. **Haymer, D. S., and J. L. Marsh.** 1986. Germ line and somatic instability of a white mutation in *Drosophila mauritiana* due to a transposable genetic element. Dev Genet **6:**281-91.
410. **Heath, R. J., J. Li, G. E. Roland, and C. O. Rock.** 2000. Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. J Biol Chem **275:**4654-9.
411. **Heath, R. J., N. Su, C. K. Murphy, and C. O. Rock.** 2000. The enoyl-[acyl-carrier-protein] reductases FabI and FabL from *Bacillus subtilis*. J Biol Chem **275:**40128-33.
412. **Heath, R. J., Y. T. Yu, M. A. Shapiro, E. Olson, and C. O. Rock.** 1998. Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis. J Biol Chem **273:**30316-20.
413. **Heathman, L. S., G. O. Pierce, and P. Kabler.** 1936. Resistance of various strains of *E. typhi* and *coli aerogenes* to chlorine and chloramine. Public Health Rep **51:**1367–87.
414. **Hechler, U., M. van den Weghe, H. H. Martin, and J. M. Frere.** 1989. Overproduced beta-lactamase and the outer-membrane barrier as resistance factors in *Serratia marcescens* highly resistant to beta-lactamase-stable beta-lactam antibiotics. J Gen Microbiol **135:**1275-90.
415. **Hedges, R. W.** 1980. R factors of *Serratia*, p. 139-53. *In* A. von Graevenitz and S. J. Rubin (ed.), The genus *Serratia*. CRC Press, Inc, Boca Raton, FL.
416. **Hedges, R. W., V. Rodriguez-Lemoine, and N. Datta.** 1975. R factors from *Serratia marcescens*. J Gen Microbiol **86:**88-92.
417. **Hefferan, M.** 1903-1904. Zentralbl Bakteriol Parasitenkd Infektionskr Abt. **11:**311-17; 397-404; 456-75; 520-40
418. **Heifets, L. B., and P. J. Lindholm-Levy.** 1990. MICs and MBCs of Win 57273 against *Mycobacterium avium* and *M. tuberculosis*. Antimicrob Agents Chemother **34:**770-4.
419. **Heinzel, M., and H. Bellinger.** 1981. Microbiological investigations with decentral dosing apparatuses for disinfectants. Part 1: investigations of cell number (author's transl). Zentralbl Bakteriol Mikrobiol Hyg **174:**299-313.
420. **Heir, E., G. Sundheim, and A. L. Holck.** 1999. Identification and characterization of quaternary ammonium compound resistant staphylococci from the food industry. Int J Food Microbiol **48:**211-9.

421. **Heir, E., G. Sundheim, and A. L. Holck.** 1999. The *qacG* gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry. J Appl Microbiol **86:**378-88.
422. **Heir, E., G. Sundheim, and A. L. Holck.** 1995. Resistance to quaternary ammonium compounds in *Staphylococcus* spp. isolated from the food industry and nucleotide sequence of the resistance plasmid pST827. J Appl Bacteriol **79:**149-56.
423. **Heir, E., G. Sundheim, and A. L. Holck.** 1998. The *Staphylococcus qacH* gene product: a new member of the SMR family encoding multidrug resistance. FEMS Microbiol Lett **163:**49-56.
424. **Hejazi, A., H. M. Aucken, and F. R. Falkiner.** 2000. Epidemiology and susceptibility of *serratia marcescens* in a large general hospital over an 8-year period. J Hosp Infect **45:**42-6.
425. **Hejazi, A., and F. R. Falkiner.** 1997. *Serratia marcescens*. J Med Microbiol **46:**903-12.
426. **Heltberg, O., F. Skov, P. Gerner-Smidt, H. J. Kolmos, E. Dybkjaer, E. Gutschik, D. Jerne, O. B. Jepsen, M. Weischer, W. Frederiksen, and *et al.*** 1993. Nosocomial epidemic of *Serratia marcescens* septicemia ascribed to contaminated blood transfusion bags. Transfusion **33:**221-7.
427. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc Natl Acad Sci USA **85:**6602-6.
428. **Henning, U., H. D. Royer, R. M. Teather, I. Hindennach, and C. P. Hollenberg.** 1979. Cloning of the structural gene (*ompA*) for an integral outer membrane protein of *Escherichia coli* K-12. Proc Natl Acad Sci USA **76:**4360-4.
429. **Hentzer, M., K. Riedel, T. B. Rasmussen, A. Heydorn, J. B. Andersen, M. R. Parsek, S. A. Rice, L. Eberl, S. Molin, N. Hoiby, S. Kjelleberg, and M. Givskov.** 2002. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiology **148:**87-102.
430. **Hentzer, M., H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, N. Kumar, M. A. Schembri, Z. Song, P. Kristoffersen, and *et al.*** 2003. Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. Embo J **22:**3803-15.
431. **Hernandez, S. M., G. P. Morlock, W. R. Butler, J. T. Crawford, and R. C. Cooksey.** 1999. Identification of *Mycobacterium* species by PCR-restriction fragment length polymorphism analyses using fluorescence capillary electrophoresis. J Clin Microbiol **37:**3688-92.
432. **Hevia, C., M. A. Bajo, J. A. Sanchez-Tomero, G. del Peso, A. Fernandez-Perpen, I. Millan, A. Aguilera, and R. Selgas.** 2000. Peritoneal catheter exit-site infections caused by rapidly-growing atypical mycobacteria. Nephrol Dial Transplant **15:**1458-60.
433. **Heym, B., P. M. Alzari, N. Honore, and S. T. Cole.** 1995. Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. Mol Microbiol **15:**235-45.
434. **Heym, B., N. Honore, C. Truffot-Pernot, A. Banerjee, C. Schurra, W. R. Jacobs, Jr., J. D. van Embden, J. H. Grosset, and S. T. Cole.** 1994. Implications of multidrug resistance for the future of short-course chemotherapy of tuberculosis: a molecular study. Lancet **344:**293-8.
435. **Higginbottom, C., S. M. Jones, and M. M. Taylor.** 1964. The influence of a change in farm dairy practice on the bacterial flora of fresh and stored raw milk. J Appl Bacteriol **27:**385-391.
436. **Hihara, Y., M. Muramatsu, K. Nakamura, and K. Sonoike.** 2004. A cyanobacterial gene encoding an ortholog of pirin is induced under stress conditions. FEBS Lett **574:**101-5.
437. **Hill, L. R.** 1966. An index to deoxyribonucleic acid base compositions of bacterial species. J Gen Microbiol **44:**419-37.
438. **Hiom, S. J., J. R. Furr, and A. D. Russell.** 1995. Uptake of 14C-chlorhexidine gluconate by *Saccharomyces cerevisiae*, *Candida albicans* and *Candida glabrata*. Lett Appl Microbiol **21:**20-2.
439. **Hiom, S. J., J. R. Furr, A. D. Russell, and J. R. Dickinson.** 1993. Effects of chlorhexidine diacetate and cetylpyridinium chloride on whole cells and protoplasts of *Saccharomyces cerevisiae*. Microbios **74:**111-20.
440. **Hiramatsu, K.** 2001. Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. Lancet Infect Dis **1:**147-55.
441. **Hoang, T. T., and H. P. Schweizer.** 1999. Characterization of *Pseudomonas aeruginosa* enoyl-acyl carrier protein reductase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis. J Bacteriol **181:**5489-97.

442. **Hogbom, M., P. Stenmark, N. Voevodskaya, G. McClarty, A. Graslund, and P. Nordlund.** 2004. The radical site in chlamydial ribonucleotide reductase defines a new R2 subclass. Science **305**:245-8.
443. **Hogman, C. F., H. Fritz, and L. Sandberg.** 1993. Posttransfusion *Serratia marcescens* septicemia. Transfusion **33**:189-91.
444. **Holden, I., I. Swift, and I. Williams.** 2000. New signal molecules on the quorum-sensing block. Trends Microbiol **8**:101-4.
445. **Holden, M. T., S. Ram Chhabra, R. de Nys, P. Stead, N. J. Bainton, P. J. Hill, M. Manefield, N. Kumar, M. Labatte, D. England, and *et al.*** 1999. Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. Mol Microbiol **33**:1254-66.
446. **Holloway, P. M., R. A. Bucknall, and G. W. Denton.** 1986. The effects of sub-lethal concentrations of chlorhexidine on bacterial pathogenicity. J Hosp Infect **8**:39-46.
447. **Holme, T.** 1972. Influence of environment on the content and composition of bacterial envelopes. J Appl Chem Biotechnol **22**:391-99.
448. **Holton, J., P. Nye, and V. McDonald.** 1994. Efficacy of selected disinfectants against mycobacteria and cryptosporidia. J Hosp Infect **27**:105-15.
449. **Horinouchi, S., and T. Beppu.** 1992. Autoregulatory factors and communication in actinomycetes. Annu Rev Microbiol **46**:377-98.
450. **Horng, Y. T., S. C. Deng, M. Daykin, P. C. Soo, J. R. Wei, K. T. Luh, S. W. Ho, S. Swift, H. C. Lai, and P. Williams.** 2002. The LuxR family protein SpnR functions as a negative regulator of *N*-acylhomoserine lactone-dependent quorum sensing in *Serratia marcescens*. Mol Microbiol **45**:1655-71.
451. **Horsburgh, C. R., Jr., and R. M. Selik.** 1989. The epidemiology of disseminated nontuberculous mycobacterial infection in the acquired immunodeficiency syndrome (AIDS). Am Rev Respir Dis **139**:4-7.
452. **Howard, S. T., and T. F. Byrd.** 2000. The rapidly growing mycobacteria: saprophytes and parasites. Microbes Infect **2**:1845-53.
453. **Howell, D.** 1972. Survey on mastitis caused by environmental bacteria. Vet Rec **90**:654-7.
454. **Hu, W. S., P. C. Li, and C. Y. Cheng.** 2005. Correlation between ceftriaxone resistance of *Salmonella enterica* serovar *Typhimurium* and expression of outer membrane proteins OmpW and Ail/OmpX-like protein, which are regulated by BaeR of a two-component system. Antimicrob Agents Chemother **49**:3955-8.
455. **Huang, A., H. Fan, W. R. Taylor, and J. A. Wright.** 1997. Ribonucleotide reductase R2 gene expression and changes in drug sensitivity and genome stability. Cancer Res **57**:4876-81.
456. **Hugo, W. B.** 1999. Disinfection mechanisms p. 258-83. *In* W. B. Hugo, A. D. Russell and G. A. J. Ayliffe (ed.), Principles and practice of disinfection, preservation and sterilization, 3rd ed. Blackwell Science, Oxford, England.
457. **Hugo, W. B.** 1992. Disinfection mechanisms. *In* W. B. Hugo, A. D. Russell and G. A. J. Ayliffe (ed.), Principles and practice of disinfection, preservation and sterilization. Blackwell Science, Oxford, England.
458. **Hugo, W. B.** 1967. The mode of action of antibacterial agents. J Appl Bacteriol **30**:17-50.
459. **Hugo, W. B.** 1979. Phenols: a review of their history and development as antimicrobial agents. Microbios **23**:83-85.
460. **Hugo, W. B., and S. F. Bloomfield.** 1971. Studies on the mode of action of the phenolic antibacterial agent fentichlor against *Staphylococcus aureus* and *Escherichia coli*. 3. The effect of fentichlor on the metabolic activities of *Staphylococcus aureus* and *Escherichia coli*. J Appl Bacteriol **34**:579-91.
461. **Hugo, W. B., and S. P. Denyer.** 1987. The concentration exponent of disinfectants and preservatives (biocides), p. 281-91. *In* R. G. Board, M. C. Allwood and J. G. Banks (ed.), Preservatives in the food, pharmaceutical and environmental industries. Blackwell Scientific Publications, Oxford. England.
462. **Hugo, W. B., and A. R. Longworth.** 1965. Cytological aspects of the mode of action of chlorhexidine diacetate. J Pharm Pharmacol **17**:28-32.
463. **Hugo, W. B., and A. R. Longworth.** 1964. Some aspects of the mode of action of chlorhexidine. J Pharm Pharmacol **16**:655-62.

464. **Hugo, W. B., and A. D. Russell.** 1999. Types of antimicrobial agents, p. 5-94. *In* A. D. Russell, W. B. Hugo and G. A. J. Ayliffe (ed.), Principles and practice of disinfectation, preservation and sterilization. Blackwell Science, Oxford, England.
465. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect Immun **33:**933-8.
466. **Hultgren, S. J., S. Abraham, M. Caparon, P. Falk, J. W. St Geme, 3rd, and S. Normark.** 1993. Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. Cell **73:**887-901.
467. **Hultgren, S. J., C. H. Jones, and S. Normark.** 1996. Bacterial adhesins and their assembly, p. 2730-56. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella* cellular and molecular biology 2nd ed. ASM Press, Washington, DC.
468. **Hultgren, S. J., F. Lindberg, G. Magnusson, J. Kihlberg, J. M. Tennent, and S. Normark.** 1989. The PapG adhesin of uropathogenic *Escherichia coli* contains separate regions for receptor binding and for the incorporation into the pilus. Proc Natl Acad Sci USA **86:**4357-61.
469. **Hung, D. L., S. D. Knight, R. M. Woods, J. S. Pinkner, and S. J. Hultgren.** 1996. Molecular basis of two subfamilies of immunoglobulin-like chaperones. Embo J **15:**3792-805.
470. **Hunter, S. W., B. Rivoire, V. Mehra, B. R. Bloom, and P. J. Brennan.** 1990. The major native proteins of the leprosy bacillus. J Biol Chem **265:**14065-8.
471. **Hurta, R. A., and J. A. Wright.** 1992. Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. J Biol Chem **267:**7066-71.
472. **Hutsul, J. A., and E. Worobec.** 1994. Molecular characterization of a 40 kDa OmpC-like porin from *Serratia marcescens*. Microbiology **140:**379-87.
473. **Hutsul, J. A., and E. Worobec.** 1997. Molecular characterization of the *Serratia marcescens* OmpF porin, and analysis of *S. marcescens* OmpF and OmpC osmoregulation. Microbiology **143:**2797-806.
474. **Ikeda, T., A. Ledwith, C. H. Bamford, and R. A. Hann.** 1984. Interaction of a polymeric biguanide biocide with phospholipid membranes. Biochim Biophys Acta **769:**57-66.
475. **Imlay, J. A., and S. Linn.** 1988. DNA damage and oxygen radical toxicity. Science **240:**1302-9.
476. **Ingram, C. W., D. C. Tanner, D. T. Durack, G. W. Kernodle, Jr., and G. R. Corey.** 1993. Disseminated infection with rapidly growing mycobacteria. Clin Infect Dis **16:**463-71.
477. **Ingram, M., and R. B. Haines.** 1949. Inhibition of bacterial growth by pure ozone in the presence of nutrients. J Hyg **47:**146-58.
478. **Inman, P. M., A. Beck, A. E. Brown, and J. L. Stanford.** 1969. Outbreak of injection abscesses due to *Mycobacterium abscessus*. Arch Dermatol **100:**141-7.
479. **Irizarry, L., T. Merlin, J. Rupp, and J. Griffith.** 1996. Reduced susceptibility of methicillin-resistant *Staphylococcus aureus* to cetylpyridinium chloride and chlorhexidine. Chemotherapy **42:**248-52.
480. **Ismaeel, N., T. el-Moug, J. R. Furr, and A. D. Russell.** 1986. Resistance of *Providencia stuartii* to chlorhexidine: a consideration of the role of the inner membrane. J Appl Bacteriol **60:**361-7.
481. **Ismaeel, N., J. R. Furr, and A. D. Russell.** 1986. Reversal of the surface effects of chlorhexidine diacetate on cells of *Providencia stuartii*. J Appl Bacteriol **61:**373-81.
482. **Ito, H., Y. Arakawa, S. Ohsuka, R. Wacharotayankun, N. Kato, and M. Ohta.** 1995. Plasmid-mediated dissemination of the metallo-beta-lactamase gene *blaIMP* among clinically isolated strains of *Serratia marcescens*. Antimicrob Agents Chemother **39:**824-9.
483. **Jack, D. L., N. M. Yang, and M. H. Saier, Jr.** 2001. The drug/metabolite transporter superfamily. Eur J Biochem **268:**3620-39.
484. **Jackson, C. G. J., and M. Fulton.** 1976. J Wildlife Dis **6:**466-68.
485. **Jackson, J. A.** 1991. Baculovirus expression systems and insect cells. Bioprocess Technol **13:**402-13.
486. **Jackson, M. W., E. Silva-Herzog, and G. V. Plano.** 2004. The ATP-dependent ClpXP and Lon proteases regulate expression of the *Yersinia pestis* type III secretion system via regulated proteolysis of YmoA, a small histone-like protein. Mol Microbiol **54:**1364-78.
487. **Jackson, P. G., H. Keen, C. J. Noble, and N. A. Simmons.** 1981. Injection abscesses due to *Mycobacterium chelonei* occurring in a diabetic patient. Tubercle **62:**277-9.

488. **Jacobs, W. A.** 1916. The bactericidal properties of the quaternary salts of hexamethylenetetramine. I. The problem of the chemotherapy of experimental bacterial infections. J Exp Med **23:**563–68.
489. **Jacobs, W. A., M. Heidelberger, and H. L. Amoss.** 1916. The bactericidal properties of the quaternary salts of hexamethylenetetramine. II. The relation between constitution and bactericidal action in the substituted benzylhexamethylenetetraminium salts. J Exp Med **23:**569–76.
490. **Jacobs, W. A., M. Heidelberger, and C. G. Bull.** 1916. The bactericidal properties of the quaternary salts of hexamethylenetetramine. III. The relation between constitution and bactericidal action in the quaternary salts obtained from halogenacetyl compounds. J Exp Med **23:**577–99.
491. **Janota-Bassalik, L.** 1963. Growth of psychrophilic and mesophilic strains of peat bacteria. Acta Microbiol Pol **12:**41-53.
492. **Jarlier, V., and H. Nikaido.** 1994. Mycobacterial cell wall: structure and role in natural resistance to antibiotics. FEMS Microbiol Lett **123:**11-8.
493. **Jarlier, V., and H. Nikaido.** 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J Bacteriol **172:**1418-23.
494. **Jessop, H. L., and P. A. Lambert.** 1986. The role of surface polysaccharide in determining the resistance of *Serratia marcescens* to serum killing. J Gen Microbiol **132:**2505-14.
495. **Jiang, W., and S. P. Schwendeman.** 2000. Formaldehyde-mediated aggregation of protein antigens: comparison of untreated and formalinized model antigens. Biotechnol Bioeng **70:**507-17.
496. **Johnson, J. S., J. Croall, J. S. Power, and G. R. Armstrong.** 1998. Fatal *Serratia marcescens* meningitis and myocarditis in a patient with an indwelling urinary catheter. J Clin Pathol **51:**789-90.
497. **Joiner, K. A., N. Grossman, M. Schmetz, and L. Leive.** 1986. C3 binds preferentially to long-chain lipopolysaccharide during alternative pathway activation by *Salmonella montevideo*. J Immunol **136:**710-5.
498. **Jones, B. V., R. Young, E. Mahenthiralingam, and D. J. Stickler.** 2004. Ultrastructure of *Proteus mirabilis* swarmer cell rafts and role of swarming in catheter-associated urinary tract infection. Infect Immun **72:**3941-50.
499. **Jones, C. H., J. S. Pinkner, A. V. Nicholes, L. N. Slonim, S. N. Abraham, and S. J. Hultgren.** 1993. FimC is a periplasmic PapD-like chaperone that directs assembly of type 1 pili in bacteria. Proc Natl Acad Sci USA **90:**8397-401.
500. **Jones, D. S., S. P. Gorman, D. F. McCafferty, and A. D. Woolfson.** 1991. The effects of three non-antibiotic, antimicrobial agents on the surface hydrophobicity of certain micro-organisms evaluated by different methods. J Appl Bacteriol **71:**218-27.
501. **Jones, M. V., T. M. Herd, and H. J. Christie.** 1989. Resistance of *Pseudomonas aeruginosa* to amphoteric and quaternary ammonium biocides. Microbios **58:**49-61.
502. **Jones, R. D., H. B. Jampani, J. L. Newman, and A. S. Lee.** 2000. Triclosan: a review of effectiveness and safety in health care settings. Am J Infect Control **28:**184-96.
503. **Jordan, A., I. Gibert, and J. Barbe.** 1995. Two different operons for the same function: comparison of the *Salmonella typhimurium nrdAB* and *nrdEF* genes. Gene **167:**75-9.
504. **Jordan, A., E. Pontis, M. Atta, M. Krook, I. Gibert, J. Barbe, and P. Reichard.** 1994. A second class I ribonucleotide reductase in *Enterobacteriaceae*: characterization of the *Salmonella typhimurium* enzyme. Proc Natl Acad Sci USA **91:**12892-6.
505. **Jordan, A., and P. Reichard.** 1998. Ribonucleotide reductases. Annu Rev Biochem **67:**71-98.
506. **Joswick, H. L., T. R. Corner, J. N. Silvernale, and P. Gerhardt.** 1971. Antimicrobial actions of hexachlorophene: release of cytoplasmic materials. J Bacteriol **108:**492-500.
507. **Kaatz, G. W., and S. M. Seo.** 1995. Inducible NorA-mediated multidrug resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother **39:**2650-5.
508. **Kaaya, G. P., and N. Darji.** 1989. Mortality in adult tsetse, Glossina morsitans morsitans, caused by entomopathogenic bacteria. J Invertebr Pathol **54:**32-8.
509. **Kadohama, N., and J. A. McCarter.** 1971. Inhibition of DNA polymerase of *E. coli* by proflavine. Can J Biochem **50:**901–8.
510. **Kagle, J., and A. Hay.** 2001. Abstract A-154. The ASM 101st general meeting, Orlando. FL.
511. **Kaiser, G. E.** July 29, 2002, posting date. TSA plate culture of the chromogenic bacterium *Serratia marcescens*. http://student.ccbcmd.edu/courses/bio141/labmanua/lab2/sminsol.html.

512. **Kalapos, M. P.** 1999. Methylglyoxal in living organisms: chemistry, biochemistry, toxicology and biological implications. Toxicol Lett **110:**145-75.
513. **Kalbe, C., P. Marten, and G. Berg.** 1996. Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. Microbiol Res **151:**433-9.
514. **Kamarudin, M. I., L. K. Fox, C. T. Gaskins, and J. M. Gay.** 1996. Environmental reservoirs for *Serratia marcescens* intramammary infections in dairy cows. J Am Vet Med Assoc **208:**555-8.
515. **Kaplan, G., R. R. Gandhi, D. E. Weinstein, W. R. Levis, M. E. Patarroyo, P. J. Brennan, and Z. A. Cohn.** 1987. *Mycobacterium leprae* antigen-induced suppression of T cell proliferation in vitro. J Immunol **138:**3028-34.
516. **Kaplan, R. W., and M. Brendel.** 1969. Formation of prototrophs in mixtures of two auxotrophic mutants of *Serratia marcescens* HY by a transducing bacteriophage produced by some auxotrophs. Mol Gen Genet **104:**27-39.
517. **Kapur, V., L. L. Li, M. R. Hamrick, B. B. Plikaytis, T. M. Shinnick, A. Telenti, W. R. Jacobs, Jr., A. Banerjee, S. Cole, K. Y. Yuen, and *et al.*** 1995. Rapid *Mycobacterium* species assignment and unambiguous identification of mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis* by automated DNA sequencing. Arch Pathol Lab Med **119:**131-8.
518. **Kapur, V., L. L. Li, S. Iordanescu, M. R. Hamrick, A. Wanger, B. N. Kreiswirth, and J. M. Musser.** 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase beta subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. J Clin Microbiol **32:**1095-8.
519. **Kar, A. K., A. S. Ghosh, K. Chauhan, J. Ahamed, J. Basu, P. Chakrabarti, and M. Kundu.** 1997. Involvement of a 43-kilodalton outer membrane protein in beta-lactam resistance of *Shigella dysenteriae*. Antimicrob Agents Chemother **41:**2302-4.
520. **Katoch, V. M., and R. A. Cox.** 1986. Step-wise isolation of RNA and DNA from mycobacteria. Int J Lepr Other Mycobact Dis **54:**409-15.
521. **Kaulfers, P.-M., and D. Brandt.** 1987. Isolation of a conjugative plasmid in *Escherichia coli* determining formaldehyde resistance. FEMS Microbiol Lett **43:**161–3.
522. **Kaulfers, P. M., and A. Wollman.** 1998. Cloning and expression of formaldehyde resistance from *Escherichia coli*. FEMS Microbiol Lett **55:**299–302.
523. **Kaulfers, P. M., and D. Brandt.** 1987. Isolation of a conjugative plasmid in *Escherichia coli* determining formaldehyde resistance. FEMS Microbiol Lett **43:**161–3.
524. **Kaulfers, P. M., H. Karch, and R. Laufs.** 1987. Plasmid-mediated formaldehyde resistance in *Serratia marcescens* and *Escherichia coli*: alterations in the cell surface. Zentralbl Bakteriol Mikrobiol Hyg **266:**239-48.
525. **Kaulfers, P. M., and R. Laufs.** 1985. Transmissible formaldehyde resistance in *Serratia marcescens*. Zentralbl Bakteriol Mikrobiol Hyg **181:**309-19.
526. **Kaulfers, P. M., and A. Wollmann.** 1988. Cloning and expression of formaldehyde resistance from *Escherichia coli*. FEMS Microbiol Lett **55:**299–302.
527. **Kawata, Y., K. Hongo, K. Nosaka, Y. Furutsu, T. Mizobata, and J. Nagai.** 1995. The role of ATP hydrolysis in the function of the chaperonin GroEL: dynamic complex formation with GroES. FEBS Lett **369:**283-6.
528. **Kay, D., and P. Fildes.** 1962. Hydroxymethylcytosine-containing and tryptophan-dependent bacteriophages isolated from city effluents. J Gen Microbiol **27:**143-6.
529. **Kazama, H., H. Hamashima, M. Sasatsu, and T. Arai.** 1998. Distribution of the antiseptic-resistance gene *qacE* delta 1 in gram-positive bacteria. FEMS Microbiol Lett **165:**295-9.
530. **Ke, T., L. Zhangfu, G. Qing, T. Yong, J. Hong, R. Hongyan, L. Kun, and L. Shigui.** 2005. Isolation of *Serratia marcescens* as a chondroitinase-producing bacterium and purification of a novel chondroitinase AC. Biotechnol Lett **27:**489-93.
531. **Kelsey, J. C.** 1967. Use of gaseous antimicrobial agents with special reference to ethylene oxide. J Appl Bacteriol **30:**92-100.
532. **Kelsey, J. C., I. H. Mackinnon, and I. M. Maurer.** 1974. Sporicidal activity of hospital disinfectants. J Clin Pathol **27:**632-8.
533. **Kenney, T. J., and G. Churchward.** 1994. Cloning and sequence analysis of the *rpsL* and *rpsG* genes of *Mycobacterium smegmatis* and characterization of mutations causing resistance to streptomycin. J Bacteriol **176:**6153-6.

534. **Kereluk, K., R. A. Gammon, and R. S. Lloyd.** 1970. Microbiological aspects of ethylene oxide sterilization. II. Microbial resistance to ethylene oxide. Appl Microbiol **19:**152-6.
535. **Keyer, K., A. S. Gort, and J. A. Imlay.** 1995. Superoxide and the production of oxidative DNA damage. J Bacteriol **177:**6782-90.
536. **Khor, S. Y., and M. Jegathesan.** 1983. Heavy metal and disinfectant resistance in clinical isolates of gram-negative rods. Southeast Asian J Trop Med Public Health **14:**199-203.
537. **Khunkitti, W., S. V. Avery, D. Lloyd, J. R. Furr, and A. D. Russell.** 1997. Effects of biocides on *Acanthamoeba castellanii* as measured by flow cytometry and plaque assay. J Antimicrob Chemother **40:**227-33.
538. **Kido, N., and H. Kobayashi.** 2000. A single amino acid substitution in a mannosyltransferase, WbdA, converts the *Escherichia coli* O9 polysaccharide into O9a: generation of a new O-serotype group. J Bacteriol **182:**2567-73.
539. **Kido, N., T. Sugiyama, T. Yokochi, H. Kobayashi, and Y. Okawa.** 1998. Synthesis of *Escherichia coli* O9a polysaccharide requires the participation of two domains of WbdA, a mannosyltransferase encoded within the *wb** gene cluster. Mol Microbiol **27:**1213-21.
540. **Kido, N., V. I. Torgov, T. Sugiyama, K. Uchiya, H. Sugihara, T. Komatsu, N. Kato, and K. Jann.** 1995. Expression of the O9 polysaccharide of *Escherichia coli*: sequencing of the *E. coli* O9 *rfb* gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. J Bacteriol **177:**2178-87.
541. **Kido, Y., H. Kodama, F. Uraki, M. Uyeda, M. Tsuruoka, and M. Shibata.** 1988. Microbial degradation of disinfectants. II. Complete degradation of chlorhexidine. Jpn J Toxicol Environ Health **34:**97-101
542. **Kikuchi, S., D. L. Rainwater, and P. E. Kolattukudy.** 1992. Purification and characterization of an unusually large fatty acid synthase from *Mycobacterium tuberculosis* var. bovis BCG. Arch Biochem Biophys **295:**318-26.
543. **Kim, J. G., A. E. Yousef, and S. Dave.** 1999. Application of ozone for enhancing the microbiological safety and quality of foods: a review. J Food Prot **62:**1071-87.
544. **Kim, J. H., E. H. Cho, K. S. Kim, H. Y. Kim, and Y. M. Kim.** 1998. Cloning and nucleotide sequence of the DNA gyrase *gyrA* gene from *Serratia marcescens* and characterization of mutations in *gyrA* of quinolone-resistant clinical isolates. Antimicrob Agents Chemother **42:**190-3.
545. **Kim, S., K. R. Willison, and A. L. Horwich.** 1994. Cystosolic chaperonin subunits have a conserved ATPase domain but diverged polypeptide-binding domains. Trends Biochem Sci **19:**543-8.
546. **Klann, A. G., A. E. Belanger, A. Abanes-De Mello, J. Y. Lee, and G. F. Hatfull.** 1998. Characterization of the *dnaG* locus in *Mycobacterium smegmatis* reveals linkage of DNA replication and cell division. J Bacteriol **180:**65-72.
547. **Klemm, P.** 1986. Two regulatory fim genes, *fimB* and *fimE*, control the phase variation of type 1 fimbriae in *Escherichia coli*. Embo J **5:**1389-93.
548. **Klemm, P., and K. A. Krogfelt.** 1994. Type 1 fimbriae of *Escherichia coli,* p. 9-26. *In* P. Klemm (ed.), Fimbriae. CRC Press Inc.
549. **Klemperer, R. M., N. T. Ismail, and M. R. Brown.** 1980. Effect of R-plasmid RP1 and nutrient depletion on the resistance of *Escherichia coli* to cetrimide, chlorhexidine and phenol. J Appl Bacteriol **48:**349-57.
550. **Klena, J. D., and C. A. Schnaitman.** 1993. Function of the *rfb* gene cluster and the *rfe* gene in the synthesis of O antigen by *Shigella dysenteriae* 1. Mol Microbiol **9:**393-402.
551. **Klose, M., H. Schwarz, S. MacIntyre, R. Freudl, M. L. Eschbach, and U. Henning.** 1988. Internal deletions in the gene for an *Escherichia coli* outer membrane protein define an area possibly important for recognition of the outer membrane by this polypeptide. J Biol Chem **263:**13291-6.
552. **Knaysi, G.** 1932. The toxicity of iodine for the cells of *Mycobacterium tuberculosis* J Infect Dis **50:**253.
553. **Koebnik, R.** 1999. Structural and functional roles of the surface-exposed loops of the beta-barrel membrane protein OmpA from *Escherichia coli*. J Bacteriol **181:**3688-94.
554. **Kolberg, M., K. R. Strand, P. Graff, and K. K. Andersson.** 2004. Structure, function, and mechanism of ribonucleotide reductases. Biochim Biophys Acta **1699:**1-34.

555. **Korner, R. J., A. Nicol, D. S. Reeves, A. P. MacGowan, and J. Hows.** 1994. Ciprofloxacin resistant *Serratia marcescens* endocarditis as a complication of non-Hodgkin's lymphoma. J Infect **29:**73-6.
556. **Kourilsky, R., R. Pieron, S. Kourilsky, R. Robineaux, and G. Voisin.** 1955. Research on the mechanism of immune-adherence phenomenon. Ann Inst Pasteur **89:**273-9.
557. **Kowalczykowski, S. C., D. A. Dixon, A. K. Eggleston, S. D. Lauder, and W. M. Rehrauer.** 1994. Biochemistry of homologous recombination in *Escherichia coli*. Microbiol Rev **58:**401-65.
558. **Kroger, A.** 1978. Fumarate as terminal acceptor of phosphorylative electron transport. Biochim Biophys Acta **505:**129–45
559. **Kroll, R. G., and G. D. Anagnostopoulos.** 1981. Potassium leakage as a lethality index of phenol and the effect of solute and water activity. J Appl Bacteriol **50:**139–47.
560. **Krulwich, T. A., P. G. Quirk, and A. A. Guffanti.** 1990. Uncoupler-resistant mutants of bacteria. Microbiol Rev **54:**52-65.
561. **Kubica, G. P., I. Baess, R. E. Gordon, P. A. Jenkins, J. B. Kwapinski, C. McDurmont, S. R. Pattyn, H. Saito, V. Silcox, J. L. Stanford, and *et al.*** 1972. A co-operative numerical analysis of rapidly growing mycobacteria. J Gen Microbiol **73:**55-70.
562. **Kubitschek, H. E.** 1969. Growth during the bacterial cell cycle: analysis of cell size distribution. Biophys J **9:**792-809.
563. **Kucken, D., H. Feucht, and P. Kaulfers.** 2000. Association of *qacE* and *qacEΔ1* with multiple resistance to antibiotics and antiseptics in clinical isolates of Gram-negative bacteria. FEMS Microbiol Lett **183:**95-8.
564. **Kuehn, M. J., S. Normark, and S. J. Hultgren.** 1991. Immunoglobulin-like PapD chaperone caps and uncaps interactive surfaces of nascently translocated pilus subunits. Proc Natl Acad Sci USA **88:**10586-90.
565. **Kuga, A., R. Okamoto, and M. Inoue.** 2000. *ampR* gene mutations that greatly increase class C beta-lactamase activity in *Enterobacter cloacae*. Antimicrob Agents Chemother **44:**561-7.
566. **Kumar, A., and E. A. Worobec.** 2005. Cloning, sequencing, and characterization of the SdeAB multidrug efflux pump of *Serratia marcescens*. Antimicrob Agents Chemother **49:**1495-501.
567. **Kumar, A., and E. A. Worobec.** 2002. Fluoroquinolone resistance of *Serratia marcescens*: involvement of a proton gradient-dependent efflux pump. J Antimicrob Chemother **50:**593-6.
568. **Kumar, A., and E. A. Worobec.** 2005. HasF, a TolC-homolog of *Serratia marcescens*, is involved in energy-dependent efflux. Can J Microbiol **51:**497-500.
569. **Kummerle, N., H. H. Feucht, and P. M. Kaulfers.** 1996. Plasmid-mediated formaldehyde resistance in *Escherichia coli*: characterization of resistance gene. Antimicrob Agents Chemother **40:**2276-9.
570. **Kundsin, R. B., and C. W. Walter.** 1957. Investigations on adsorption of benzalkonium chloride U.S.P. by skin, gloves, and sponges. AMA Arch Surg **75:**1036-42.
571. **Kundu, M., J. Basu, and P. Chakrabarti.** 1991. Defective mycolic acid biosynthesis in a mutant of *Mycobacterium smegmatis*. J Gen Microbiol **137:**2197-200.
572. **Kunito, T., T. Kusano, H. Oyaizu, K. Senoo, S. Kanazawa, and S. Matsumoto.** 1996. Cloning and sequence analysis of *czc* genes in *Alcaligenes* sp. strain CT14. Biosci Biotechnol Biochem **60:**699-704.
573. **Kusunoki, S., and T. Ezaki.** 1992. Proposal of *Mycobacterium peregrinum* sp. nov., nom. rev., and elevation of *Mycobacterium chelonae* subsp. *abscessus* (Kubica *et al.*) to species status: *Mycobacterium abscessus* comb. nov. Int J Syst Bacteriol **42:**240-5.
574. **Kuyyakanond, T., and L. B. Quesnel.** 1992. The mechanism of action of chlorhexidine. FEMS Microbiol Lett **79:**211-5.
575. **Labbate, M., S. Y. Queck, K. S. Koh, S. A. Rice, M. Givskov, and S. Kjelleberg.** 2004. Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. J Bacteriol **186:**692-8.
576. **Lambert, P. A.** 1988. *Enterobacteriaceae*: composition, structure and function of the cell envelope. Soc Appl Bacteriol Symp Ser **17:**21S-34S.
577. **Lambert, P. A.** 2004. Mechanisms of action of biocides p. 139-53. *In* A. P. Fraise, P. A. Lambert and J-Y. Maillard (ed.), Principles and practice of disinfectation, preservation and sterilization, 4th ed. Blackwell Publishing, Oxford, England.

578. **Lambert, P. A., and S. M. Hammond.** 1973. Potassium fluxes, first indications of membrane damage in micro-organisms. Biochem Biophys Res Commun **54:**796-9.
579. **Lancaster, C. R., A. Kroger, M. Auer, and H. Michel.** 1999. Structure of fumarate reductase from *Wolinella succinogenes* at 2.2 A resolution. Nature **402:**377-85.
580. **Lancaster, L. J.** 1962. Role of *Serratia* species in urinary tract infections. Arch Intern Med **109:**536-9.
581. **Langsrud, S., T. Moretro, and G. Sundheim.** 2003. Characterization of *Serratia marcescens* surviving in disinfecting footbaths. J Appl Microbiol **95:**186-95.
582. **Lannigan, R., and L. E. Bryan.** 1985. Decreased susceptibility of *Serratia marcescens* to chlorhexidine related to the inner membrane. J Antimicrob Chemother **15:**559-65.
583. **Lapik, Y. R., and L. S. Kaufman.** 2003. The *Arabidopsis* cupin domain protein AtPirin1 interacts with the G protein alpha-subunit GPA1 and regulates seed germination and early seedling development. Plant Cell **15:**1578-90.
584. **Latifi, A., M. K. Winson, M. Foglino, B. W. Bycroft, G. S. Stewart, A. Lazdunski, and P. Williams.** 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Mol Microbiol **17:**333-43.
585. **Laurent, E.** 1890. Ann Inst Pasteur Paris **4:**465-83
586. **Lazdunski, A. M., I. Ventre, and J. N. Sturgis.** 2004. Regulatory circuits and communication in Gram-negative bacteria. Nat Rev Microbiol **2:**581-92.
587. **Le Minor, L., C. Coynault, and M. Schwartz.** 1974. Plasmid determined lactose positive character in "*Serratia liquefaciens*". Ann Microbiol **125B:**357-66.
588. **Lear, J. C., J. Y. Maillard, P. W. Dettmar, P. A. Goddard, and A. D. Russell.** 2002. Chloroxylenol- and triclosan-tolerant bacteria from industrial sources. J Ind Microbiol Biotechnol **29:**238-42.
589. **Lederer, E., A. Adam, R. Ciorbaru, J. F. Petit, and J. Wietzerbin.** 1975. Cell walls of *Mycobacteria* and related organisms; chemistry and immunostimulant properties. Mol Cell Biochem **7:**87-104.
590. **Lee, R. E., J. W. Armour, K. Takayama, P. J. Brennan, and G. S. Besra.** 1997. Mycolic acid biosynthesis: definition and targeting of the Claisen condensation step. Biochim Biophys Acta **1346:**275-84.
591. **Lee, R. E., P. J. Brennan, and G. S. Besra.** 1996. *Mycobacterium tuberculosis* cell envelope. Curr Top Microbiol Immunol **215:**1-27.
592. **Leelaporn, A., I. T. Paulsen, J. M. Tennent, T. G. Littlejohn, and R. A. Skurray.** 1994. Multidrug resistance to antiseptics and disinfectants in coagulase-negative staphylococci. J Med Microbiol **40:**214-20.
593. **Leers, W. D.** 1980. Disinfecting endoscopes: how not to transmit *Mycobacterium tuberculosis* by bronchoscopy. Can Med Assoc J **123:**275-80.
594. **Lehmann, K. B., and Neumann R.** 1896. Atlas und Grundriss der Bakteriologie und Lehrbuch der speciellen bakteriologischen Diagnostik. J.F.Lehmann, München **1.**
595. **Lehmann, K. B., and Neumann R.** 1907. Lehmann's Medizin, Handatlanten. X. Atlas und Grundriss der Bakteriologie und Lehrbuch der Speziellen Bakteriologischen Diagnostik. 4 Auflage. J.F. Lehmann, Munchen: p. 500.
596. **Lehmann, K. B., and Neumann R.** 1899. Lehmann's Medizin, Handatlanter X. Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik.
597. **Leminor, L., C. Coynault, and M. Schwartz.** 1974. Determination plasmidique du caractere "lactose positif" de *Serratia liquefaciens*. Ann Microbiol **125B:**357-66.
598. **Lemosquet-Villemon, M., C. Morel, and F. Freymuth.** 1973. Gentamycin resistance. Pathol Biol **21:**227-32.
599. **Leranoz, S., P. Orus, M. Berlanga, F. Dalet, and M. Vinas.** 1997. New fimbrial adhesins of *Serratia marcescens* isolated from urinary tract infections: description and properties. J Urol **157:**694-8.
600. **Lerouge, I., and J. Vanderleyden.** 2002. O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. FEMS Microbiol Rev **26:**17-47.
601. **Levy, S. B.** 2002. Active efflux, a common mechanism for biocide and antibiotic resistance. J Appl Microbiol **92 (Suppl):**65S-71S.

602. **Lewenza, S., B. Conway, E. P. Greenberg, and P. A. Sokol.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J Bacteriol **181:**748-56.
603. **Lewis, K., D. C. Hooper, and M. Ouellette.** 1997. Multidrug resistance pumps provide broad defense. ASM News **63:**605-10.
604. **Li, L., T. Komatsu, A. Inoue, and K. Horikoshi.** 1995. A toluene-tolerant mutant of *Pseudomonas aeruginosa* lacking the outer membrane protein F. Biosci Biotechnol Biochem **59:**2358-9.
605. **Li, X. Z., and H. Nikaido.** 2004. Efflux-mediated drug resistance in bacteria. Drugs **64:**159-204.
606. **Li, X. Z., L. Zhang, and H. Nikaido.** 2004. Efflux pump-mediated intrinsic drug resistance in *Mycobacterium smegmatis*. Antimicrob Agents Chemother **48:**2415-23.
607. **Liau, S. Y., D. C. Read, W. J. Pugh, J. R. Furr, and A. D. Russell.** 1997. Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. Lett Appl Microbiol **25:**279-83.
608. **Liebeskind, D. S., N. Ostrzega, C. G. Wasterlain, and E. A. Buttner.** 2001. Neurologic manifestations of disseminated infection with *Mycobacterium abscessus*. Neurology **56:**810-3.
609. **Limansky, A. S., M. A. Mussi, and A. M. Viale.** 2002. Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. J Clin Microbiol **40:**4776-8.
610. **Lindberg, F., J. M. Tennent, S. J. Hultgren, B. Lund, and S. Normark.** 1989. PapD, a periplasmic transport protein in P-pilus biogenesis. J Bacteriol **171:**6052-8.
611. **Lion, T., and O. A. Haas.** 1990. Nonradioactive labeling of probe with digoxigenin by polymerase chain reaction. Anal Biochem **188:**335-7.
612. **Littlejohn, T. G., D. DiBeradino, L. J. Messerotti, S. J. Spiers, and R. A. Skurray.** 1990. Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. Gene **101:**59–66.
613. **Littlejohn, T. G., I. T. Paulsen, M. T. Gillespie, J. M. Tennent, M. Midgley, I. G. Jones, A. S. Purewal, and R. A. Skurray.** 1992. Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*. FEMS Microbiol Lett **74:**259-65.
614. **Littlejohn, T. G., D. A. Rouch, D. S. Cram, D. Di Berardino, and R. A. Skurray.** 1991. Molecular and sequence analysis of QacA specifying antiseptic-disinfectant resistance in staphylococci, p. 175-82. *In* R. P. Novick (ed.), Molecular biology of the staphylococci. VCH Publishers, Inc, New York.
615. **Liu, J., and H. Nikaido.** 1999. A mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids accumulates meromycolates. Proc Natl Acad Sci USA **96:**4011-6.
616. **Liu, J., E. Y. Rosenberg, and H. Nikaido.** 1995. Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. Proc Natl Acad Sci USA **92:**11254-8.
617. **Liu, J., H. E. Takiff, and H. Nikaido.** 1996. Active efflux of fluoroquinolones in *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump. J Bacteriol **178:**3791-5.
618. **Llorca, O., J. L. Carrascosa, and J. M. Valpuesta.** 1996. Biochemical characterization of symmetric GroEL-GroES complexes. Evidence for a role in protein folding. J Biol Chem **271:**68-76.
619. **Llorca, O., S. Marco, J. L. Carrascosa, and J. M. Valpuesta.** 1994. The formation of symmetrical GroEL-GroES complexes in the presence of ATP. FEBS Lett **345:**181-6.
620. **Loffer, E. D.** 1990. Disinfection vs. sterilization of gynecologic laparoscopy equipment. The experience of the Phoenix Surgicenter. J Reprod Med **25:**263–6.
621. **Lorimer, G. H.** 1996. A quantitative assessment of the role of the chaperonin proteins in protein folding *in vivo*. Faseb J **10:**5-9.
622. **Loshon, C. A., P. C. Genest, B. Setlow, and P. Setlow.** 1999. Formaldehyde kills spores of *Bacillus subtilis* by DNA damage and small, acid-soluble spore proteins of the alpha/beta-type protect spores against this DNA damage. J Appl Microbiol **87:**8-14.
623. **Loughlin, M. F., M. V. Jones, and P. A. Lambert.** 2002. *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. J Antimicrob Chemother **49:**631-9.
624. **Lowbury, E. J., and H. A. Lilly.** 1973. Use of 4 per cent chlorhexidine detergent solution (Hibiscrub) and other methods of skin disinfection. Br Med J **1:**510-5.

625. **Lowry, P. W., C. M. Beck-Sague, L. A. Bland, S. M. Aguero, M. J. Arduino, A. N. Minuth, R. A. Murray, J. M. Swenson, and W. R. Jarvis.** 1990. *Mycobacterium chelonae* infection among patients receiving high-flux dialysis in a hemodialysis clinic in California. J Infect Dis **161:**85-90.
626. **Lucchini, J. J., J. Corre, and A. Cremieux.** 1990. Antibacterial activity of phenolic compounds and aromatic alcohols. Res Microbiol **141:**499-510.
627. **Lucchini, S., G. Rowley, M. D. Goldberg, D. Hurd, M. Harrison, and J. C. Hinton.** 2006. H-NS mediates the silencing of laterally acquired genes in bacteria. PLoS Pathog **2:**e81.
628. **Lugtenberg, B., and L. van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim Biophys Acta **737:**51-115.
629. **Lustgarten, S.** 1885. The bacillus of syphilis. Lancet **i:**609-10.
630. **Luzzaro, F., M. Perilli, R. Migliavacca, G. Lombardi, P. Micheletti, A. Agodi, S. Stefani, G. Amicosante, and L. Pagani.** 1998. Repeated epidemics caused by extended-spectrum beta-lactamase-producing *Serratia marcescens* strains. Eur J Clin Microbiol Infect Dis **17:**629-36.
631. **Lynam, P. A., J. R. Babb, and A. P. Fraise.** 1995. Comparison of the mycobactericidal activity of 2% alkaline glutaraldehyde and 'Nu-Cidex' (0.35% peracetic acid). J Hosp Infect **30:**237-40.
632. **Lyon, B. R., J. L. Iuorio, J. W. May, and R. A. Skurray.** 1984. Molecular epidemiology of multiresistant *Staphylococcus aureus* in Australian hospitals. J Med Microbiol **17:**79-89.
633. **Lyon, B. R., J. W. May, and R. A. Skurray.** 1984. Tn*4001*: a gentamicin and kanamycin resistance transposon in *Staphylococcus aureus*. Mol Gen Genet **193:**554-6.
634. **Lyon, B. R., and R. Skurray.** 1987. Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. Microbiol Rev **51:**88-134.
635. **Lysenko, O.** 1976. Chitinase of *Serratia marcescens* and its toxicity to insects. J Invertebr Pathol **27:**385-6.
636. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol Microbiol **16:**45-55.
637. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1993. Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J Bacteriol **175:**6299-313.
638. **Ma, D., D. N. Cook, J. E. Hearst, and H. Nikaido.** 1994. Efflux pumps and drug resistance in gram-negative bacteria. Trends Microbiol **2:**489-93.
639. **Mace, E.** 1901. Traite Pratique de Bacteriologic, Paris: Bailliere.
640. **MacGowan, A. P., and R. Wise.** 2001. Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. J Antimicrob Chemother **48 (Suppl 1):**17-28.
641. **MacIntyre, S., R. Lucken, and P. Owen.** 1986. Smooth lipopolysaccharide is the major protective antigen for mice in the surface extract from IATS serotype 6 contributing to the polyvalent *Pseudomonas aeruginosa* vaccine PEV. Infect Immun **52:**76-84.
642. **Madhusudan, S., A. Paukner, Y. Klingen, and K. Schnetz.** 2005. Independent regulation of H-NS-mediated silencing of the *bgl* operon at two levels: upstream by BglJ and LeuO and downstream by DnaKJ. Microbiology **151:**3349-59.
643. **Madrid, C., C. Balsalobre, J. Garcia, and A. Juarez.** 2007. The novel Hha/YmoA family of nucleoid-associated proteins: use of structural mimicry to modulate the activity of the H-NS family of proteins. Mol Microbiol **63:**7-14.
644. **Mahenthiralingam, E., M. E. Campbell, J. Foster, J. S. Lam, and D. P. Speert.** 1996. Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. J Clin Microbiol **34:**1129-35.
645. **Mahenthiralingam, E., M. E. Campbell, D. A. Henry, and D. P. Speert.** 1996. Epidemiology of *Burkholderia cepacia* infection in patients with cystic fibrosis: analysis by randomly amplified polymorphic DNA fingerprinting. J Clin Microbiol **34:**2914-20.
646. **Maillard, J. Y.** 2002. Bacterial target sites for biocide action. Symp Ser Soc Appl Microbiol **92:**16S-27S.
647. **Maillard, J. Y., and A. D. Russell.** 1997. Viricidal activity and mechanisms of action of biocides. Sci Prog **80:**287-315.

648. **Maki, D. G., C. G. Hennekens, C. W. Phillips, W. V. Shaw, and J. V. Bennett.** 1973. Nosocomial urinary tract infection with *Serratia marcescens*: an epidemiologic study. J Infect Dis **128:**579-87.

649. **Mallick, S. A.** 1996. Cell surface hydrophobicity and its relation to outer membrane proteins of *Serratia marcescens*. Indian J Exp Biol **34:**107-10.

650. **Maloney, S., S. Welbel, B. Daves, K. Adams, S. Becker, L. Bland, M. Arduino, R. Wallace, Jr., Y. Zhang, G. Buck, and *et al.*** 1994. *Mycobacterium abscessus* pseudoinfection traced to an automated endoscope washer: utility of epidemiologic and laboratory investigation. J Infect Dis **169:**1166-9.

651. **Malouin, F., G. D. Campbell, M. Halpenny, G. W. Becker, and T. R. Parr, Jr.** 1990. Outer membrane and porin characteristics of *Serratia marcescens* grown in vitro and in rat intraperitoneal diffusion chambers. Infect Immun **58:**1247-53.

652. **Mammeri, H., L. Poirel, P. Bemer, H. Drugeon, and P. Nordmann.** 2004. Resistance to cefepime and cefpirome due to a 4-amino-acid deletion in the chromosome-encoded AmpC beta-lactamase of a *Serratia marcescens* clinical isolate. Antimicrob Agents Chemother **48:**716-20.

653. **Mandel, M., and R. Rownd.** 1964. Deoxyribonucleic acid base composition in the *Enterobacteriaceae*: an evolutionary sequence? p. 585-97. *In* C. A. Leone (ed.), Taxonomic biochemistry and serology. Ronald Press, New York.

654. **Manderville, R. A.** 2001. Synthesis, proton-affinity and anti-cancer properties of the prodigiosin-group natural products. Curr Med Chem Anticancer Agents **1:**195-218.

655. **Manoil, C.** 2000. Tagging exported proteins using *Escherichia coli* alkaline phosphatase gene fusions. Methods Enzymol **326:**35-47.

656. **Manzoor, S. E., P. A. Lambert, P. A. Griffiths, M. J. Gill, and A. P. Fraise.** 1999. Reduced glutaraldehyde susceptibility in *Mycobacterium chelonae* associated with altered cell wall polysaccharides. J Antimicrob Chemother **43:**759-65.

657. **Marcinkeviciene, J., W. Jiang, L. M. Kopcho, G. Locke, Y. Luo, and R. A. Copeland.** 2001. Enoyl-ACP reductase (FabI) of *Haemophilus influenzae*: steady-state kinetic mechanism and inhibition by triclosan and hexachlorophene. Arch Biochem Biophys **390:**101-8.

658. **Maris, P.** 1995. Modes of action of disinfectants. Rev Sci Tech **14:**47-55.

659. **Markovic, M., and M. Radojicic.** 1996. Studies of digestive tract bacterial flora in crucian carp (*Carassius carssius*). Veterinarki Glasnik **50:**911-14.

660. **Marques, S., M. Manzanera, M. M. Gonzalez-Perez, M. T. Gallegos, and J. L. Ramos.** 1999. The XylS-dependent Pm promoter is transcribed *in vivo* by RNA polymerase with sigma 32 or sigma 38 depending on the growth phase. Mol Microbiol **31:**1105-13.

661. **Marre, R., J. Hacker, and V. Braun.** 1989. The cell-bound hemolysin of *Serratia marcescens* contributes to uropathogenicity. Microb Pathog **7:**153-6.

662. **Marrie, T. J., and J. W. Costerton.** 1981. Prolonged survival of *Serratia marcescens* in chlorhexidine. Appl Environ Microbiol **42:**1093-102.

663. **Martinez-Martinez, L., A. Pascual, M. C. Conejo, L. Picabea, and E. J. Perea.** 1999. Resistance of *Pseudomonas aeruginosa* to imipenem induced by eluates from siliconized latex urinary catheters is related to outer membrane protein alterations. Antimicrob Agents Chemother **43:**397-9.

664. **Masecar, B. L., and N. J. Robillard.** 1991. Spontaneous quinolone resistance in *Serratia marcescens* due to a mutation in *gyrA*. Antimicrob Agents Chemother **35:**898-902.

665. **Maseda, H., H. Yoneyama, and T. Nakae.** 2000. Assignment of the substrate-selective subunits of the MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **44:**658-64.

666. **Matsumoto, H., T. Tazaki, and S. Hosogaya.** 1973. A generalized transducing phage of *Serratia marcescens*. Jpn J Microbiol **17:**473-9.

667. **Matsumoto, H., T. Tazaki, and S. Hosogaya.** 1974. Transductional analysis of the leu-thr region of chromosome of *Serratia marcescens*. J Bacteriol **117:**1365-7.

668. **Mayo, M. S., R. L. Schlitzer, M. A. Ward, L. A. Wilson, and D. G. Ahearn.** 1987. Association of *Pseudomonas* and *Serratia* corneal ulcers with use of contaminated solutions. J Clin Microbiol **25:**1398-400.

669. **Mayville, P., G. Ji, R. Beavis, H. Yang, M. Goger, R. P. Novick, and T. W. Muir.** 1999. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc Natl Acad Sci USA **96:**1218-23.
670. **McBain, A. J., A. H. Rickard, and P. Gilbert.** 2002. Possible implications of biocide accumulation in the environment on the prevalence of bacterial antibiotic resistance. J Ind Microbiol Biotechnol **29:**326-30.
671. **McCallum, K. L., G. Schoenhals, D. Laakso, B. Clarke, and C. Whitfield.** 1989. A high-molecular-weight fraction of smooth lipopolysaccharide in *Klebsiella* serotype O1:K20 contains a unique O-antigen epitope and determines resistance to nonspecific serum killing. Infect Immun **57:**3816-22.
672. **McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, and *et al.*** 1997. Quorum sensing and chromobacterium violaceum: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiology **143:**3703-11.
673. **McCormack, R. C., and C. M. Kunin.** 1966. Control of a single source nursery epidemic due to *Serratia marcescens*. Pediatrics **37:**750-5.
674. **McCracken, A. W., and F. E. Lipscomb.** 1965. *Serratia marcescens* infection complicating peritoneal dialysis. Br Med J **1:**1536-7.
675. **McDermott, P. F., D. G. White, I. Podglajen, M. N. Alekshun, and S. B. Levy.** 1998. Multidrug resistance following expression of the *Escherichia coli marA* gene in *Mycobacterium smegmatis*. J Bacteriol **180:**2995-8.
676. **McDonnell, G., and A. D. Russell.** 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev **12:**147-79.
677. **McElhaney, R. N., J. de Gier, and E. C. van der Neut-Kok.** 1973. The effect of alterations in fatty acid composition and cholesterol content on the nonelectrolyte permeability of *Acholeplasma laidlawii* B cells and derived liposomes. Biochim Biophys Acta **298:**500-12.
678. **McGowan, S. J., A. M. Barnard, G. Bosgelmez, M. Sebaihia, N. J. Simpson, N. R. Thomson, D. E. Todd, M. Welch, N. A. Whitehead, and G. P. Salmond.** 2005. Carbapenem antibiotic biosynthesis in *Erwinia carotovora* is regulated by physiological and genetic factors modulating the quorum sensing-dependent control pathway. Mol Microbiol **55:**526-45.
679. **McGowan, S. J., B. W. Bycroft, and G. P. Salmond.** 1998. Bacterial production of carbapenems and clavams: evolution of beta-lactam antibiotic pathways. Trends Microbiol **6:**203-8.
680. **McIntosh, D., and B. Austin.** 1990. Recovery of an extremely proteolytic form of *Serratia liquefaciens* as a pathogen of Atlantic salmon, Salmo salar, in Scotland. J Fish Biol **36:**765-72.
681. **McKenna, S. M., and K. J. Davies.** 1988. The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. Biochem J **254:**685-92.
682. **McLeod, R., S. P. Muench, J. B. Rafferty, D. E. Kyle, E. J. Mui, M. J. Kirisits, D. G. Mack, C. W. Roberts, B. U. Samuel, R. E. Lyons, and *et al.*** 2001. Triclosan inhibits the growth of *Plasmodium falciparum* and *Toxoplasma gondii* by inhibition of apicomplexan Fab I. Int J Parasitol **31:**109-13.
683. **McMurry, L. M., P. F. McDermott, and S. B. Levy.** 1999. Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for triclosan. Antimicrob Agents Chemother **43:**711-3.
684. **McMurry, L. M., M. Oethinger, and S. B. Levy.** 1998. Overexpression of *marA, soxS,* or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. FEMS Microbiol Lett **166:**305-9.
685. **McMurry, L. M., M. Oethinger, and S. B. Levy.** 1998. Triclosan targets lipid synthesis. Nature **394:**531-2.
686. **McNab, R., S. K. Ford, A. El-Sabaeny, B. Barbieri, G. S. Cook, and R. J. Lamont.** 2003. LuxS-based signaling in *Streptococcus gordonii:* autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. J Bacteriol **185:**274-84.
687. **McNaughton, M., N. Mazinke, and E. Thomas.** 1995. Newborn conjunctivitis associated with triclosan 0.5% antiseptic intrinsically contaminated with *Serratia marcescens*. Can J Infect Control **10:**7-8.
688. **McNeil, M., M. Daffe, and P. J. Brennan.** 1991. Location of the mycolyl ester substituents in the cell walls of mycobacteria. J Biol Chem **266:**13217-23.

689. **Meade, M. J., R. L. Waddell, and T. M. Callahan.** 2001. Soil bacteria *Pseudomonas putida* and *Alcaligenes xylosoxidans* subsp. denitrificans inactivate triclosan in liquid and solid substrates. FEMS Microbiol Lett **204:**45-8.

690. **Mechin, L., F. Dubois-Brissonnet, B. Heyd, and J. Y. Leveau.** 1999. Adaptation of *Pseudomonas aeruginosa* ATCC 15442 to didecyldimethylammonium bromide induces changes in membrane fatty acid composition and in resistance of cells. J Appl Microbiol **86:**859-66.

691. **Medeiros, A. A., and T. F. O'Brien.** 1969. Contribution of R factors to the antibiotic resistance of hospital isolates of *Serratia*. Antimicrob Agents Chemother **8:**30–35.

692. **Meier-Dieter, U., R. Starman, K. Barr, H. Mayer, and P. D. Rick.** 1990. Biosynthesis of enterobacterial common antigen in *Escherichia coli*. Biochemical characterization of *Tn*10 insertion mutants defective in enterobacterial common antigen synthesis. J Biol Chem **265:**13490-7.

693. **Meincke, B. E., R. G. Kranz, and D. L. Lynch.** 1980. Effect of irgasan on bacterial growth and its adsorption into the cell wall. Microbios **28:**133-47.

694. **Meltz, D. J., and M. H. Grieco.** 1973. Characteristics of *Serratia marcescens* pneumonia. Arch Intern Med **132:**359-64.

695. **Meredith, F. T., and D. J. Sexton.** 1996. *Mycobacterium abscessus* osteomyelitis following a plantar puncture wound. Clin Infect Dis **23:**651-3.

696. **Metcalf, J. F., J. F. John, Jr., G. B. Wilson, H. H. Fudenberg, and R. A. Harley.** 1981. *Mycobacterium fortuitum* pulmonary infection associated with an antigen-selective defect in cellular immunity. Am J Med **71:**485-92.

697. **Meyers, H., B. A. Brown-Elliott, D. Moore, J. Curry, C. Truong, Y. Zhang, and R. J. Wallace Jr.** 2002. An outbreak of *Mycobacterium chelonae* infection following liposuction. Clin Infect Dis **34:**1500-7.

698. **Miall, S. H., and I. O. Walker.** 1966. Structural studies on ribosomes I. The binding of proflavine to *Escherichia coli* ribosomes. Biochemica and Biophysica Acta **145:**82–95.

699. **Midelfart, J., A. Midelfart, and L. Bevanger.** 1996. Microbial contamination of contact lens cases among medical students. Clao J **22:**21-4.

700. **Miesel, L., T. R. Weisbrod, J. A. Marcinkeviciene, R. Bittman, and W. R. Jacobs, Jr.** 1998. NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in *Mycobacterium smegmatis*. J Bacteriol **180:**2459-67.

701. **Mikulskis, A. V., and G. R. Cornelis.** 1994. A new class of proteins regulating gene expression in enterobacteria. Mol Microbiol **11:**77-86.

702. **Mikulskis, A. V., I. Delor, V. H. Thi, and G. R. Cornelis.** 1994. Regulation of the *Yersinia enterocolitica* enterotoxin Yst gene. Influence of growth phase, temperature, osmolarity, pH and bacterial host factors. Mol Microbiol **14:**905-15.

703. **Miller, P. F., L. F. Gambino, M. C. Sulavik, and S. J. Gracheck.** 1994. Genetic relationship between *soxRS* and mar loci in promoting multiple antibiotic resistance in *Escherichia coli*. Antimicrob Agents Chemother **38:**1773-9.

704. **Miller, P. F., and M. C. Sulavik.** 1996. Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli*. Mol Microbiol **21:**441-8.

705. **Miller, S. M.** 1999. Bacterial detoxification of Hg(II) and organomercurials. Essays Biochem **34:**17-30.

706. **Miner, N. A., J. W. McDowell, G. W. Willcockson, N. I. Bruckner, R. L. Stark, and E. J. Whitmore.** 1977. Antimicrobial and other properties of a new stabilized alkaline glutaraldehyde disinfectant/sterilizer. Am J Hosp Pharm **34:**376-82.

707. **Minnikin, D. E.** 1982. Lipids: complex lipids, their chemistry, biosynthesis, and roles, p. 95-184. *In* C. Ratledge and J. S. Stanford (ed.), The biology of mycobacteria. Academic Press, London, England.

708. **Minnikin, D. E.** 1991. Chemical principles in the organization of lipid components in the mycobacterial cell envelope. Res Microbiol **142:**423-7.

709. **Minnikin, D. E., H. Abdolrahimzadeh, and J. Baddiley.** 1971. The interrelation of phosphatidylethanolamine and glycosyl diglycerides in bacterial membranes. Biochem J **124:**447-8.

710. **Miquel, P., and R. Cambier.** 1902. Traite de bacteriologie pure et appliquee a la medecine et a I'hygiene, Paris: Naud.

711. **Misaki, A., N. Seto, and I. Azuma.** 1974. Structure and immunological properties of D-arabino-D-galactans isolated from cell walls of *Mycobacterium* species. J Biochem **76:**15-27.
712. **Misra, T. K.** 1992. Bacterial resistances to inorganic mercury salts and organomercurials. Plasmid **27:**4-16.
713. **Mitchell, B. A., M. H. Brown, and R. A. Skurray.** 1998. QacA multidrug efflux pump from *Staphylococcus aureus*: comparative analysis of resistance to diamidines, biguanidines, and guanylhydrazones. Antimicrob Agents Chemother **42:**475-7.
714. **Mitchell, P.** 1972. Chemiosmotic coupling in energy transduction: a logical development of biochemical knowledge. J Bioenerg **3:**5-24.
715. **Mitchell, P.** 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature **191:**144-8.
716. **Mizuguchi, Y., T. Udou, and T. Yamada.** 1983. Mechanism of antibiotic resistance in *Mycobacterium intracellulare*. Microbiol Immunol **27:**425-31.
717. **Mizunoe, Y., T. Matsumoto, M. Haraoka, M. Sakumoto, S. Kubo, and J. Kumazawa.** 1995. Effect of pili of *Serratia marcescens* on superoxide production and phagocytosis of human polymorphonuclear leukocytes. J Urol **154:**1227-30.
718. **Mojica, F. J., and C. F. Higgins.** 1997. *In vivo* supercoiling of plasmid and chromosomal DNA in an *Escherichia coli hns* mutant. J Bacteriol **179:**3528-33.
719. **Moken, M. C., L. M. McMurry, and S. B. Levy.** 1997. Selection of multiple-antibiotic-resistant (*mar*) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. Antimicrob Agents Chemother **41:**2770-2.
720. **Moore, J. S., M. Christensen, R. W. Wilson, R. J. Wallace, Jr., Y. Zhang, D. R. Nash, and B. Shelton.** 2000. Mycobacterial contamination of metalworking fluids: involvement of a possible new taxon of rapidly growing mycobacteria. Aihaj **61:**205-13.
721. **Moore, M., and J. B. Frerichs.** 1953. An unusual acid-fast infection of the knee with subcutaneous, abscess-like lesions of the gluteal region; report of a case with a study of the organism, *Mycobacterium abscessus*, n. sp. J Invest Dermatol **20:**133-69.
722. **Moore, S. L., and D. N. Payne.** 2004. Types of antimicrobial agents, p. 8-97. *In* A. P. Fraise, P. A. Lambert and J-Y. Maillard (ed.), Principles and practice of disinfectation, preservation and sterilization, 4th ed. Blackwell Publishing, Oxford, England.
723. **Morona, R., M. Klose, and U. Henning.** 1984. *Escherichia coli* K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. J Bacteriol **159:**570-8.
724. **Morona, R., L. van den Bosch, and P. A. Manning.** 1995. Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. J Bacteriol **177:**1059-68.
725. **Morris, E. J., and H. M. Darlow.** 1971. Inactivation of viruses, p. 687–702. *In* W. B. Hugo (ed.), Inhibition and destruction of the microbial Cell. Academic Press, London, England.
726. **Morrissey, A. T., and D. G. Fraenkel.** 1968. Selection of fructose 6-phosphate kinase mutants in *Escherichia coli*. Biochem Biophys Res Commun **32:**467-73.
727. **Morse, L. J., and L. E. Schonbeck.** 1968. Hand lotions--a potential nosocomial hazad. N Engl J Med **278:**376-8.
728. **Morton, H. E.** 1950. The relationship of concentration and germicidal efficiency of ethyl alcohol. Ann N Y Acad Sci **53:**191-6.
729. **Mourino, M., C. Madrid, C. Balsalobre, A. Prenafeta, F. Munoa, J. Blanco, M. Blanco, J. E. Blanco, and A. Juarez.** 1996. The Hha protein as a modulator of expression of virulence factors in *Escherichia coli*. Infect Immun **64:**2881-4.
730. **Mourino, M., F. Munoa, C. Balsalobre, P. Diaz, C. Madrid, and A. Juarez.** 1994. Environmental regulation of alpha-haemolysin expression in *Escherichia coli*. Microb Pathog **16:**249-59.
731. **Movva, R. N., K. Nakamura, and M. Inouye.** 1980. Amino acid sequence of the signal peptide of OmpA protein, a major outer membrane protein of *Escherichia coli*. J Biol Chem **225:**27-29.
732. **Movva, R. N., K. Nakamura, and M. Inouye.** 1980. Gene structure of the OmpA protein, a major surface protein of *Escherichia coli* required for cell-cell interaction. J Mol Biol **142:**317-28.
733. **Muir, M. E., R. S. van Heeswyck, and B. J. Wallace.** 1984. Effect of growth rate on streptomycin accumulation by *Escherichia coli* and *Bacillus megaterium*. J Gen Microbiol **130:**2015-22.

734. **Mukhopadhyay, S., D. Basu, and P. Chakrabarti.** 1997. Characterization of a porin from *Mycobacterium smegmatis*. J Bacteriol **179:**6205-7.
735. **Munch-Petersen, A., and B. Mygind.** 1976. Nucleoside transport systems in *Escherichia coli* K12: specificity and regulation. J Cell Physiol **89:**551-9.
736. **Munch-Petersen, A., P. Nygaard, K. Hammer-Jespersen, and N. Fiil.** 1972. Mutants constitutive for nucleoside-catabolizing enzymes in *Escherichia coli* K12. Isolation, charactrization and mapping. Eur J Biochem **27:**208-15.
737. **Murata-Kamiya, N., and H. Kamiya.** 2001. Methylglyoxal, an endogenous aldehyde, crosslinks DNA polymerase and the substrate DNA. Nucleic Acids Res **29:**3433-8.
738. **Murillo, J., J. Torres, L. Bofill, A. Rios-Fabra, E. Irausquin, R. Isturiz, M. Guzman, J. Castro, L. Rubino, and M. Cordido.** 2000. Skin and wound infection by rapidly growing mycobacteria: an unexpected complication of liposuction and liposculpture. The Venezuelan Collaborative Infectious and Tropical Diseases Study Group. Arch Dermatol **136:**1347-52.
739. **Murray, G. L., S. R. Attridge, and R. Morona.** 2003. Regulation of *Salmonella typhimurium* lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. Mol Microbiol **47:**1395-406.
740. **Muschel, L. H., and L. J. Larsen.** 1970. The sensitivity of smooth and rough gram-negative bacteria to the immune bactericidal reaction. Proc Soc Exp Biol Med **133:**345-8.
741. **Musser, J. M.** 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin Microbiol Rev **8:**496-514.
742. **Naas, T., L. Vandel, W. Sougakoff, D. M. Livermore, and P. Nordmann.** 1994. Cloning and sequence analysis of the gene for a carbapenem-hydrolyzing class A beta-lactamase, Sme-1, from *Serratia marcescens* S6. Antimicrob Agents Chemother **38:**1262-70.
743. **Nakajima, H., M. Kobayashi, T. Negishi, and R. Aono.** 1995. *soxRS* gene increased the level of organic solvent tolerance in *Escherichia coli*. Biosci Biotechnol Biochem **59:**1323-5.
744. **Nakamura, H.** 1968. Genetic determination of resistance to acriflavine, phenethyl alcohol, and sodium dodecyl sulfate in *Escherichia coli*. J Bacteriol **96:**987-96.
745. **Nakamura, K., and T. Tamaoki.** 1968. Reversible dissociation of *Escherichia coli* ribosomes by hydrogen peroxide. Biochim Biophys Acta **161:**368-76.
746. **Nakashima, A. K., M. A. McCarthy, W. J. Martone, and R. L. Anderson.** 1987. Epidemic septic arthritis caused by *Serratia marcescens* and associated with a benzalkonium chloride antiseptic. J Clin Microbiol **25:**1014-8.
747. **Naroditskaya, V., M. J. Schlosser, N. Y. Fang, and K. Lewis.** 1993. An *E. coli* gene *emrD* is involved in adaptation to low energy shock. Biochem Biophys Res Commun **196:**803-9.
748. **Navarre, W. W., S. Porwollik, Y. Wang, M. McClelland, H. Rosen, S. J. Libby, and F. C. Fang.** 2006. Selective silencing of foreign DNA with low GC content by the H-NS protein in *Salmonella*. Science **313:**236-8.
749. **Nealson, K. H., and J. W. Hastings.** 1979. Bacterial bioluminescence: its control and ecological significance. Microbiol Rev **43:**496-518.
750. **Neidle, S., and Z. Abraham.** 1984. Structural and sequence-dependent aspects of drug intercalation into nucleic acids. CRC Crit Rev Biochem **17:**73-121.
751. **Nelson, B. R., R. P. Rapini, R. J. Wallace, Jr., and J. A. Tschen.** 1989. Disseminated *Mycobacterium chelonae* ssp. *abscessus* in an immunocompetent host and with a known portal of entry. J Am Acad Dermatol **20:**909-12.
752. **Nelson, K. E., P. A. Larson, D. E. Schraufnagel, and J. Jackson.** 1983. Transmission of tuberculosis by flexible fiberbronchoscopes. Am Rev Respir Dis **127:**97-100.
753. **Neu, H. C.** 1992. The crisis in antibiotic resistance. Science **257:**1064-73.
754. **Newton, G. L., M. D. Unson, S. J. Anderberg, J. A. Aguilera, N. N. Oh, S. B. delCardayre, Y. Av-Gay, and R. C. Fahey.** 1999. Characterization of *Mycobacterium smegmatis* mutants defective in 1-d-myo-inosityl-2-amino-2-deoxy-alpha-d-glucopyranoside and mycothiol biosynthesis. Biochem Biophys Res Commun **255:**239-44.
755. **Newton, J. A., Jr., P. J. Weiss, W. A. Bowler, and E. C. Oldfield, 3rd.** 1993. Soft-tissue infection due to *Mycobacterium smegmatis*: report of two cases. Clin Infect Dis **16:**531-3.
756. **Nicas, T. I., and R. E. Hancock.** 1980. Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. J Bacteriol **143:**872-8.

757. **Nielsen, K. L., N. McLennan, M. Masters, and N. J. Cowan.** 1999. A single-ring mitochondrial chaperonin (Hsp60-Hsp10) can substitute for GroEL-GroES *in vivo*. J Bacteriol **181:**5871-5.
758. **Nikaido, H.** 1998. Antibiotic resistance caused by gram-negative multidrug efflux pumps. Clin Infect Dis **27 (Suppl 1):**S32-41.
759. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol **178:**5853-9.
760. **Nikaido, H.** 1998. Multiple antibiotic resistance and efflux. Curr Opin Microbiol **1:**516-23.
761. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substances. Biochim Biophys Acta **433:**118-32.
762. **Nikaido, H.** 1990. Permeability of the lipid domains of bacterial membranes. Adv Membr Fluid **4:**165-90.
763. **Nikaido, H.** 1994. Porins and specific diffusion channels in bacterial outer membranes. J Biol Chem **269:**3905-8.
764. **Nikaido, H.** 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. Semin Cell Dev Biol **12:**215-23.
765. **Nikaido, H., M. Basina, V. Nguyen, and E. Y. Rosenberg.** 1998. Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those beta-lactam antibiotics containing lipophilic side chains. J Bacteriol **180:**4686-92.
766. **Nikaido, H., and V. Jarlier.** 1991. Permeability of the mycobacterial cell wall. Res Microbiol **142:**437-43.
767. **Nikaido, H., S. H. Kim, and E. Y. Rosenberg.** 1993. Physical organization of lipids in the cell wall of *Mycobacterium chelonae*. Mol Microbiol **8:**1025-30.
768. **Nishida, M., Y. Mine, T. Matsubara, S. Goto, and S. Kuwahara.** 1972. Bicyclomycin, a new antibiotic. 3. *In vitro* and *in vivo* antimicrobial activity. J Antibiot **25:**582-93.
769. **Nishihara, T., T. Okamoto, and N. Nishiyama.** 2000. Biodegradation of didecyldimethylammonium chloride by *Pseudomonas fluorescens* TN4 isolated from activated sludge. J Appl Microbiol **88:**641-7.
770. **Nishino, K., and A. Yamaguchi.** 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. J Bacteriol **183:**5803-12.
771. **Nishino, K., and A. Yamaguchi.** 2002. EvgA of the two-component signal transduction system modulates production of the *yhiUV* multidrug transporter in *Escherichia coli*. J Bacteriol **184:**2319-23.
772. **Noguchi, N., M. Hase, M. Kitta, M. Sasatsu, K. Deguchi, and M. Kono.** 1999. Antiseptic susceptibility and distribution of antiseptic-resistance genes in methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol Lett **172:**247-53.
773. **Nomura, K., and T. Yoshida.** 1990. Nucleotide sequence of the *Serratia marcescens* SR50 chromosomal *ampC* beta-lactamase gene. FEMS Microbiol Lett **58:**295-9.
774. **Novitsky, J. A., and R. Y. Morita.** 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrophilic marine vibrio. Appl Environ Microbiol **32:**617-22.
775. **Nukaga, M., S. Haruta, K. Tanimoto, K. Kogure, K. Taniguchi, M. Tamaki, and T. Sawai.** 1995. Molecular evolution of a class C beta-lactamase extending its substrate specificity. J Biol Chem **270:**5729-35.
776. **Nyunoya, H., and C. J. Lusty.** 1983. The *carB* gene of *Escherichia coli*: a duplicated gene coding for the large subunit of carbamoyl-phosphate synthetase. Proc Natl Acad Sci USA **80:**4629-33.
777. **O'Connor, H. J., and A. T. Axon.** 1983. Gastrointestinal endoscopy: infection and disinfection. Gut **24:**1067-77.
778. **Ogase, H., I. Nagai, K. Kameda, S. Kume, and S. Ono.** 1992. Identification and quantitative analysis of degradation products of chlorhexidine with chlorhexidine-resistant bacteria with three-dimensional high performance liquid chromatography. J Appl Bacteriol **73:**71–78.
779. **Okuda, T., N. Endo, Y. Osada, and H. Zen-Yoji.** 1984. Outbreak of nosocomial urinary tract infections caused by *Serratia marcescens*. J Clin Microbiol **20:**691-5.
780. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J Bacteriol **178:**306-8.
781. **Olenick, J. G., and F. E. Hahn.** 1972. Mode of action of primaquine: preferential inhibition of protein biosynthesis in *Bacillus megaterium*. Antimicrob Agents Chemother **1:**259-62.

782. **Olivier, K. N., D. J. Weber, R. J. Wallace, Jr., A. R. Faiz, J. H. Lee, Y. Zhang, B. A. Brown-Elliot, A. Handler, R. W. Wilson, M. S. Schechter, and *et al.*** 2003. Nontuberculous mycobacteria. I: multicenter prevalence study in cystic fibrosis. Am J Respir Crit Care Med **167:**828-34.

783. **Orzaez, D., A. J. de Jong, and E. J. Woltering.** 2001. A tomato homologue of the human protein PIRIN is induced during programmed cell death. Plant Mol Biol **46:**459-68.

784. **Pallent, L. J., W. B. Hugo, D. J. Grant, and A. Davies.** 1983. *Pseudomonas cepacia* as contaminant and infective agent. J Hosp Infect **4:**9-13.

785. **Palomar, J., R. Montilla, M. C. Fuste, and M. Vinas.** 1993. The role of O-antigen in susceptibility of *Serratia marcescens* to non-immune serum. Microbios **76:**189-96.

786. **Palomar, J., M. Puig, R. Montilla, J. G. Loren, and M. Vinas.** 1995. Lipopolysaccharide recovery restores susceptibility levels towards beta-lactams in *Serratia marcescens*. Microbios **82:**21-6.

787. **Pandita, D., and P. J. Carson.** 1999. Thyroid abscess caused by *Mycobacterium chelonae*. Clin Infect Dis **28:**1183-4.

788. **Pao, S. S., I. T. Paulsen, and M. H. Saier, Jr.** 1998. Major facilitator superfamily. Microbiol Mol Biol Rev **62:**1-34.

789. **Papavinasasundaram, K. G., M. J. Colston, and E. O. Davis.** 1998. Construction and complementation of a *recA* deletion mutant of *Mycobacterium smegmatis* reveals that the intein in *Mycobacterium tuberculosis recA* does not affect RecA function. Mol Microbiol **30:**525-34.

790. **Parikh, S. L., G. Xiao, and P. J. Tonge.** 2000. Inhibition of InhA, the enoyl reductase from *Mycobacterium tuberculosis*, by triclosan and isoniazid. Biochemistry **39:**7645-50.

791. **Parish, T., J. Liu, H. Nikaido, and N. G. Stoker.** 1997. A *Mycobacterium smegmatis* mutant with a defective inositol monophosphate phosphatase gene homolog has altered cell envelope permeability. J Bacteriol **179:**7827-33.

792. **Park, J. B., and N. H. Park.** 1989. Effect of chlorhexidine on the in vitro and *in vivo* herpes simplex virus infection. Oral Surg Oral Med Oral Pathol **67:**149-53.

793. **Park, S. H., and A. Bendelac.** 2000. CD1-restricted T-cell responses and microbial infection. Nature **406:**788-92.

794. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu Rev Genet **26:**71-112.

795. **Parment, P. A., R. Ronnerstam, and M. Walder.** 1986. Persistence of *Serratia marcescens*, *Serratia liquefaciens* and *E. coli* in solutions for contact lenses. Acta Ophthalmol (Copenh) **64:**456-62.

796. **Patarca, R., J. A. Rosenzwei, A. A. Zuniga, and M. A. Fletcher.** 2000. Benzalkonium salts: effects on G protein-mediated processes and surface membranes. Crit Rev Oncog **11:**255-305.

797. **Pattyn, S. R., J. Vandepitte, F. Portaels, and A. De Muynck.** 1971. Cases of *Mycobacterium borstelense* and *M. abscessus* infection observed in Belgium. J Med Microbiol **4:**145-9.

798. **Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell, and R. A. Skurray.** 1996. Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity. Proc Natl Acad Sci USA **93:**3630-5.

799. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1998. Characterization of the earliest known *Staphylococcus aureus* plasmid encoding a multidrug efflux system. J Bacteriol **180:**3477-9.

800. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1996. Proton-dependent multidrug efflux systems. Microbiol Rev **60:**575-608.

801. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1999. Characterization of the earliest known *Staphylococcus aureus* plasmid encoding a multidrug efflux system. J. Bacteriol **180:**3477-9.

802. **Paulsen, I. T., T. G. Littlejohn, P. Radstrom, L. Sundstrom, O. Skold, G. Swedberg, and R. A. Skurray.** 1993. The 3' conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob Agents Chemother **37:**761-8.

803. **Paulsen, I. T., and R. A. Skurray.** 1993. Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes-an analysis. Gene **124:**1-11.

804. **Paulsen, I. T., R. A. Skurray, R. Tam, M. H. Saier, Jr., R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius.** 1996. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol Microbiol **19:**1167-75.
805. **Payne, D. N., J. R. Babb, and C. R. Bradley.** 1999. An evaluation of the suitability of the European suspension test to reflect *in vitro* activity of antiseptics against clinically significant organisms. Lett Appl Microbiol **28:**7-12.
806. **Payne, D. N., S. A. Gibson, and R. Lewis.** 1998. Antiseptics: a forgotten weapon in the control of antibiotic resistant bacteria in hospital and community settings? J R Soc Health **118:**18-22.
807. **Payne, G. W.** 2006. Molecular analysis of the diversity and pollutant tolerance of the *Burkholderia* genus. Cardiff University, Cardiff.
808. **Pearce, H., S. Messager, and J. Y. Maillard.** 1999. Effect of biocides commonly used in the hospital environment on the transfer of antibiotic-resistance genes in *Staphylococcus aureus*. J Hosp Infect **43:**101-7.
809. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc Natl Acad Sci USA **91:**197-201.
810. **Pechey, D. T., and A. M. James.** 1974. Surface properties of cells of gentamicin-sensitive and gentamicin-resistant strains of *Pseudomonas aeruginosa*. Microbios **10A (Suppl):**111-26.
811. **Peeters, A., B. Vandercam, C. J. Sindic, P. Hantson, and P. Mahieu.** 1997. Community-acquired *Serratia marcescens* meningitis. J Infect **35:**303-4.
812. **Pelicic, V., M. Jackson, J. M. Reyrat, W. R. Jacobs, Jr., B. Gicquel, and C. Guilhot.** 1997. Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA **94:**10955-60.
813. **Pereira, B. J., P. Unnykrishnan, K. S. Prasad, and R. Macaden.** 1990. An epidemic outbreak of *Serratia marcescens* septicemia in a hemodialysis unit. Ren Fail **12:**121-3.
814. **Perez-Rueda, E., and J. Collado-Vides.** 2000. The repertoire of DNA-binding transcriptional regulators in *Escherichia coli* K-12. Nucleic Acids Res **28:**1838-47.
815. **Perez-Tomas, R., and B. Montaner.** 2003. Effects of the proapoptotic drug prodigiosin on cell cycle-related proteins in Jurkat T cells. Histol Histopathol **18:**379-85.
816. **Perez-Tomas, R., B. Montaner, E. Llagostera, and V. Soto-Cerrato.** 2003. The prodigiosins, proapoptotic drugs with anticancer properties. Biochem Pharmacol **66:**1447-52.
817. **Persino, R., and D. L. Lynch.** 1982. Divalent cation dependent resistance in *E. coli* LMR-26 to the broad spectrum antimicrobial agent irgasan. Microbios **34:**41-57.
818. **Peteroy, M., A. Severin, F. Zhao, D. Rosner, U. Lopatin, H. Scherman, A. Belanger, B. Harvey, G. F. Hatfull, P. J. Brennan, and N. D. Connell.** 2000. Characterization of a *Mycobacterium smegmatis* mutant that is simultaneously resistant to D-cycloserine and vancomycin. Antimicrob Agents Chemother **44:**1701-4.
819. **Peterson, A. A., and E. J. McGroarty.** 1985. High-molecular-weight components in lipopolysaccharides of *Salmonella typhimurium, Salmonella minnesota,* and *Escherichia coli*. J Bacteriol **162:**738-45.
820. **Petit, J. F., A. Adam, J. Wietzerbin-Falszpan, E. Lederer, and J. M. Ghuysen.** 1969. Chemical structure of the cell wall of *Mycobacterium smegmatis*. I. Isolation and partial characterization of the peptidoglycan. Biochem Biophys Res Commun **35:**478-85.
821. **Petrini, B.** 2006. *Mycobacterium abscessus*: an emerging rapid-growing potential pathogen. Apmis **114:**319-28.
822. **Pettijohn, D. E.** 1988. Histone-like proteins and bacterial chromosome structure. J Biol Chem **263:**12793-6.
823. **Petty, N. K., I. J. Foulds, E. Pradel, J. J. Ewbank, and G. P. Salmond.** 2006. A generalized transducing phage (phiIF3) for the genomically sequenced *Serratia marcescens* strain Db11: a tool for functional genomics of an opportunistic human pathogen. Microbiology **152:**1701-8.
824. **Piddock, L. J.** 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev **19:**382-402.
825. **Pierard, A., N. Glansdorff, M. Mergeay, and J. M. Wiame.** 1965. Control of the biosynthesis of carbamoyl phosphate in *Escherichia coli*. J Mol Biol **14:**23-36.
826. **Pierucci, O.** 1978. Dimensions of *Escherichia coli* at various growth rates: model for envelope growth. J Bacteriol **135:**559-74.

827. **Piette, J., H. Nyunoya, C. J. Lusty, R. Cunin, G. Weyens, M. Crabeel, D. Charlier, N. Glansdorff, and A. Pierard.** 1984. DNA sequence of the *carA* gene and the control region of *carAB*: tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoyl-phosphate synthetase in *Escherichia coli* K-12. Proc Natl Acad Sci USA **81**:4134-8.

828. **Pirhonen, M., D. Flego, R. Heikinheimo, and E. T. Palva.** 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. Embo J **12**:2467-76.

829. **Platt, D. J., and J. S. Sommerville.** 1981. *Serratia* species isolated from patients in a general hospital. J Hosp Infect **2**:341-8.

830. **Platt, D. J., and J. S. Sommerville.** 1981. A simple method for the detection of resistance plasmids in *Serratia* species by transfer to members of the genus *Enterobacter*. J Antimicrob Chemother **8**:145-52.

831. **Plaus, W. J., and G. Hermann.** 1991. The surgical management of superficial infections caused by atypical mycobacteria. Surgery **110**:99-103.

832. **Poinar, G. O., Jr., H. J. Wassink, M. E. Leegwater-van der Linden, and L. P. van der Geest.** 1979. *Serratia marcescens* as a pathogen of tsetse flies. Acta Trop **36**:223-7.

833. **Poole, K.** 2004. Bacterial resistance: Acquired resistance, p. 170-83. *In* A. P. Fraise, P. A. Lambert and J-Y. Maillard (ed.), Principles and practice of disinfectation, preservation and sterilization, 4th ed. Blackwell Publishing, Oxford, England.

834. **Poole, K.** 2002. Mechanisms of bacterial biocide and antibiotic resistance. J Appl Microbiol **92 (Suppl)**:55S-64S.

835. **Poole, K.** 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J Mol Microbiol Biotechnol **3**:255-64.

836. **Popham, D. L., S. Sengupta, and P. Setlow.** 1995. Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water content and with or without major DNA-binding proteins. Appl Environ Microbiol **61**:3633-8.

837. **Porat, R., M. A. Johns, and W. R. McCabe.** 1987. Selective pressures and lipopolysaccharide subunits as determinants of resistance of clinical isolates of gram-negative bacilli to human serum. Infect Immun **55**:320-8.

838. **Prasadarao, N. V., C. A. Wass, J. N. Weiser, M. F. Stins, S. H. Huang, and K. S. Kim.** 1996. Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. Infect. Immun **64**:146–53.

839. **Preiss, J., K. Crawford, J. Downey, C. Lammel, and E. Greenberg.** 1976. Kinetic properties of *Serratia marcescens* adenosine 5'-diphosphate glucose pyrophosphorylase. J Bacteriol **127**:193-203.

840. **Prinz, B. M., S. Michaelis, N. Kettelhack, B. Mueller, G. Burg, and W. Kempf.** 2004. Subcutaneous infection with *Mycobacterium abscessus* in a renal transplant recipient. Dermatology **208**:259-61.

841. **Prinzis, S., D. Chatterjee, and P. J. Brennan.** 1993. Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG. J Gen Microbiol **139**:2649-58.

842. **Prosseda, G., M. Falconi, M. Giangrossi, C. O. Gualerzi, G. Micheli, and B. Colonna.** 2004. The *virF* promoter in *Shigella*: more than just a curved DNA stretch. Mol Microbiol **51**:523-37.

843. **Puig, M., C. Fuste, and M. Vinas.** 1993. Outer membrane proteins from *Serratia marcescens*. Can J Microbiol **39**:108-11.

844. **Pulvertaft, R. J. V., and G. D. Lumb.** 1948. Bacterial lysis and antiseptics. J Hyg **46**:62–4.

845. **Putman, M., H. W. van Veen, and W. N. Konings.** 2000. Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev **64**:672-93.

846. **Quack, J. M.** 1976. Quaternary ammonium compounds in cosmetics. Cosmet toileteries **91**:35–52.

847. **Quinn, P. J., M. E. Carter, B. K. Markey, and G. R. Carter.** 1994. *Mycobacterium* species, p. 156–9, Clinical Veterinary Microbiology. Wolfe, London

848. **Radziejewska-Lebrecht, J., D. Krajewska-Pietrasik, and H. Mayer.** 1990. Terminal and chain-linked residues of D-galacturonic acid: characteristic constituents of the R-core regions of Proteae and of *Serratia marcescens*. Syst Appl Microbiol **13**:214–9.

849. **Raetz, C. R., and C. Whitfield.** 2002. Lipopolysaccharide endotoxins. Annu Rev Biochem **71**:635-700.
850. **Rahman, M., J. Naidoo, and R. C. George.** 1988. New genetic location of gentamicin-resistance in methicillin-resistant *Staphylococcus aureus*. Lancet **2**:1256.
851. **Ramakrishnan, L., R. H. Valdivia, J. H. McKerrow, and S. Falkow.** 1997. *Mycobacterium marinum* causes both long-term subclinical infection and acute disease in the leopard frog (Rana pipiens). Infect Immun **65**:767-73.
852. **Ramos-Aires, J., P. Plesiat, L. Kocjancic-Curty, and T. Kohler.** 2004. Selection of an antibiotic-hypersusceptible mutant of *Pseudomonas aeruginosa*: identification of the GlmR transcriptional regulator. Antimicrob Agents Chemother **48**:843-51.
853. **Ramos, J. L., M. T. Gallegos, S. Marques, M. I. Ramos-Gonzalez, M. Espinosa-Urgel, and A. Segura.** 2001. Responses of Gram-negative bacteria to certain environmental stressors. Curr Opin Microbiol **4**:166-71.
854. **Rana, F. R., E. A. Macias, C. M. Sultany, M. C. Modzrakowski, and J. Blazyk.** 1991. Interactions between magainin 2 and *Salmonella typhimurium* outer membranes: effect of lipopolysaccharide structure. Biochemistry **30**:5858-66.
855. **Ranganthan, N. S.** 1996. Handbook of disinfectants and antiseptics. Marcel Dekker, Inc, New York, NY.
856. **Rastogi, N., and K. S. Goh.** 1990. Action of 1-isonicotinyl-2-palmitoyl hydrazine against the *Mycobacterium avium* complex and enhancement of its activity by m-fluorophenylalanine. Antimicrob Agents Chemother **34**:2061-4.
857. **Raulston, J. E., and T. C. Montie.** 1989. Early cell envelope alterations by tobramycin associated with its lethal action on *Pseudomonas aeruginosa*. J Gen Microbiol **135**:3023-34.
858. **Rawat, M., J. Heys, and Y. Av-Gay.** 2003. Identification and characterization of a diamide sensitive mutant of *Mycobacterium smegmatis*. FEMS Microbiol Lett **220**:161-9.
859. **Rawat, M., G. L. Newton, M. Ko, G. J. Martinez, R. C. Fahey, and Y. Av-Gay.** 2002. Mycothiol-deficient *Mycobacterium smegmatis* mutants are hypersensitive to alkylating agents, free radicals, and antibiotics. Antimicrob Agents Chemother **46**:3348-55.
860. **Rees, E. L.** 2004. The genetic basis of triclosan and chlorhexidine resistance in *Burkholderia cenocepacia*. Cardiff University, Cardiff.
861. **Regos, J., and H. R. Hitz.** 1974. Investigations on the mode of action of triclosan, a broad spectrum antimicrobial agent. Zentralbl Bakteriol **226**:390-401.
862. **Regue, M., C. Fabregat, and M. Vinas.** 1991. A generalized transducing bacteriophage for *Serratia marcescens*. Res Microbiol **142**:23-7.
863. **Reichard, P.** 1993. From RNA to DNA, why so many ribonucleotide reductases? Science **260**:1773-7.
864. **Reichard, P., A. Baldesten, and L. Rutberg.** 1961. Formation of deoxycytidine phosphates from cytidine phosphates in extracts from *Escherichia coli*. J Biol Chem **236**:1150-7.
865. **Ren, H., and J. Liu.** 2006. AsnB is involved in natural resistance of *Mycobacterium smegmatis* to multiple drugs. Antimicrob Agents Chemother **50**:250-5.
866. **Rensing, C., B. Fan, R. Sharma, B. Mitra, and B. P. Rosen.** 2000. CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. Proc Natl Acad Sci USA **97**:652-6.
867. **Repath, F., J. H. Seabury, C. V. Sanders, and J. Domer.** 1976. Prosthetic valve endocarditis due to *Mycobacterium chelonei*. South Med J **69**:1244-6.
868. **Revel, V., E. Cambau, V. Jarlier, and W. Sougakoff.** 1994. Characterization of mutations in *Mycobacterium smegmatis* involved in resistance to fluoroquinolones. Antimicrob Agents Chemother **38**:1991-6.
869. **Reverdy, M. E., M. Bes, Y. Brun, and J. Fleurette.** 1993. Evolution of resistance to antibiotics and antiseptics of hospital *Staphylococcus aureus* strains isolated from 1980 to 1991. Pathol Biol **41**:897-904.
870. **Reverdy, M. E., M. Bes, C. Nervi, A. Martra, and J. Fieurette.** 1992. Activity of four antiseptics and of ethidium bromide on 392 strains representing 26 *Staphylococcus* spp. Med Microbiol Lett **1**:56-63.
871. **Rhoades, E. R., R. Ringrose, J. A. Mohr, L. Brooks, B. A. McKown, and F. Felton.** 1971. Contamination of ultrasonic nebulization equipment with gram negative bacteria. Arch Intern Med **127**:228-32.

872. **Rice, S. A., K. S. Koh, S. Y. Queck, M. Labbate, K. W. Lam, and S. Kjelleberg.** 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. J Bacteriol **187:**3477-85.
873. **Richards, W. D., and C. O. Thoen.** 1979. Chemical destruction of *Mycobacterium bovis* in milk. J Food Prot **42:**55-7.
874. **Richardson, A.** 1970. Bovine mastitis associated with *Mycobacterium smegmatis* and an untypable *Mycobacterium*. Vet Rec **86:**497-8.
875. **Richardson, A.** 1971. The experimental production of mastitis in sheep by *Mycobacterium smegmatis* and *Mycobacterium fortuitum*. Cornell Vet **61:**640-6.
876. **Richmond, M. H., and R. B. Sykes.** 1973. The beta-lactamases of gram-negative bacteria and their possible physiological role. Adv Microb Physiol **9:**31-88.
877. **Rick, P. D., G. L. Hubbard, and K. Barr.** 1994. Role of the *rfe* gene in the synthesis of the O8 antigen in *Escherichia coli* K-12. J Bacteriol **176:**2877-84.
878. **Rickloff, J. R.** 1985. An evaluation of the sporicidal activity of ozone. Appl Environ Microbiol **53:**683–6.
879. **Riedel, K., T. Ohnesorg, K. A. Krogfelt, T. S. Hansen, K. Omori, M. Givskov, and L. Eberl.** 2001. N-acyl-L-homoserine lactone-mediated regulation of the *lip* secretion system in *Serratia liquefaciens* MG1. J Bacteriol **183:**1805-9.
880. **Rikimaru, T., M. Kondo, S. Kondo, and K. Oizumi.** 2000. Efficacy of common antiseptics against mycobacteria. Int J Tuberc Lung Dis **4:**570-6.
881. **Riley, J. G., M. Menggad, P. J. Montoya-Peleaz, W. A. Szarek, C. L. Marolda, M. A. Valvano, J. S. Schutzbach, and I. Brockhausen.** 2005. The *wbbD* gene of *E. coli* strain VW187 (O7:K1) encodes a UDP-Gal: GlcNAc{alpha}-pyrophosphate-R {beta}1,3-galactosyltransferase involved in the biosynthesis of O7-specific lipopolysaccharide. Glycobiology **15:**605-13.
882. **Ringrose, R. E., B. McKown, F. G. Felton, B. O. Barclay, H. G. Muchmore, and E. R. Rhoades.** 1968. A hospital outbreak of *Serratia marcescens* associated with ultrasonic nebulizers. Ann Intern Med **69:**719-29.
883. **Roantree, R. J., T. T. Kuo, and D. G. MacPhee.** 1977. The effect of defined lipopolysaccharide core defects upon antibiotic resistances of *Salmonella typhimurium*. J Gen Microbiol **103:**223-34.
884. **Robinson, J. P., and D. G. Fraenkel.** 1978. Allosteric and non-allosteric *E. coli* phosphofructokinases: effects on growth. Biochem Biophys Res Commun **81:**858-63.
885. **Rosner, J. L., and J. L. Slonczewski.** 1994. Dual regulation of *inaA* by the multiple antibiotic resistance (*mar*) and superoxide (*soxRS*) stress response systems of *Escherichia coli*. J Bacteriol **176:**6262-9.
886. **Rossmoore, H. W., and M. Sondossi.** 1988. Applications and mode of action of formaldehyde condensate biocides. Adv Appl Microbiol **33:**223-77.
887. **Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol Microbiol **4:**2051-62.
888. **Roussel, A., A. Lucas, and G. Bouley.** 1969. Rev Pathol Comp Med Exp **6:**27-29.
889. **Roussow, F. T., and R. J. Roussow.** 1984. Effects pf the resistance plasmid R124 on the level of OmpF outer membrane protein and on the response of *Escherichia coli* to environmental agents. J Appl Bacteriol **56:**63-79.
890. **Rubbo, S. D., J. F. Gardner, and R. L. Webb.** 1967. Biocidal activities of glutaraldehyde and related compounds. J Appl Bacteriol **30:**78-87.
891. **Rubin, E. J., B. J. Akerley, V. N. Novik, D. J. Lampe, R. N. Husson, and J. J. Mekalanos.** 1999. *In vivo* transposition of *mariner*-based elements in enteric bacteria and mycobacteria. Proc Natl Acad Sci USA **96:**1645-50.
892. **Ruiz, N., E. Maier, C. Andersen, R. Benz, and M. Vinas.** 2004. Molecular and functional characterisation of the *Serratia marcescens* outer membrane protein Ompl. Biophys Chem **109:**215-27.
893. **Runyon, E. H.** 1959. Anonymous mycobacteria in pulmonary disease. Med Clin North Am **43:**273-90.
894. **Russell, A. D.** 1996. Activity of biocides against mycobacteria. Soc Appl Bacteriol Symp Ser **25:**87S-101S.

895. **Russell, A. D.** 2002. Antibiotic and biocide resistance in bacteria: comments and conclusions. Symp Ser Soc Appl Microbiol **92:**171S-3S.
896. **Russell, A. D.** 2002. Antibiotic and biocide resistance in bacteria: introduction. Symp Ser Soc Appl Microbiol **92:**1S-3S.
897. **Russell, A. D.** 1999. Bacterial resistance to disinfectants: present knowledge and future problems. J Hosp Infect **43 (Suppl):**S57-S68.
898. **Russell, A. D.** 1990. Bacterial spores and chemical sporicidal agents. Clin Microbiol Rev **3:**99-119.
899. **Russell, A. D.** 2003. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. Lancet Infect Dis **3:**794-803.
900. **Russell, A. D.** 1986. Chlorhexidine: antibacterial action and bacterial resistance. Infection **14:**212-5.
901. **Russell, A. D.** 1971. The destruction of bacterial spores, p. 451–612. *In* W. B. Hugo (ed.), Inhibition and destruction of the microbial cell. Academic Press, London, England.
902. **Russell, A. D.** 1982. The destruction of bacterial spores. Academic Press, London, England.
903. **Russell, A. D.** 2000. Do biocides select for antibiotic resistance? J Pharm Pharmacol **52:**227-33.
904. **Russell, A. D.** 1994. Glutaraldehyde: current status and uses. Infect Control Hosp Epidemiol **15:**724-33.
905. **Russell, A. D.** 2002. Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. Symp Ser Soc Appl Microbiol **92:**121S-35S.
906. **Russell, A. D.** 2002. Mechanisms of antimicrobial action of antiseptics and disinfectants: an increasingly important area of investigation. J Antimicrob Chemother **49:**597-9.
907. **Russell, A. D.** 2001. Mechanisms of bacterial insusceptibility to biocides. Am J Infect Control **29:**259-61.
908. **Russell, A. D.** 1998. Mechanisms of bacterial resistance to antibiotics and biocides. Prog Med Chem **35:**133-97.
909. **Russell, A. D.** 1998. Microbial susceptibility and resistance to chemical and physical agents, p. 149–84. *In* A. Balows and B. I. Duerden (ed.), Topley and Wilson's microbiology and microbial infections, 9th ed, vol. 2. Edward Arnold, London, UK.
910. **Russell, A. D.** 1997. Plasmids and bacterial resistance to biocides. J Appl Microbiol **83:**155-65.
911. **Russell, A. D.** 1991. Principles of antimicrobial activity, p. 27–58. *In* S. S. Block (ed.), Disinfection, sterilization and preservation. Lea & Febinger, Philadelphia.
912. **Russell, A. D.** 2003. Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother **52:**750-63.
913. **Russell, A. D., and I. Chopra.** 1996. Understanding Antibacterial Action and Resistance, vol. 2. Ellis Horwood, Chichester.
914. **Russell, A. D., and M. J. Day.** 1993. Antibacterial activity of chlorhexidine. J Hosp Infect **25:**229-38.
915. **Russell, A. D., and M. J. Day.** 1996. Antibiotic and biocide resistance in bacteria. Microbios **85:**45-65.
916. **Russell, A. D., and J. R. Furr.** 1977. The antibacterial activity of a new chloroxylenol preparation containing ethylenediamine tetraacetic acid. J Appl Bacteriol **43:**253-60.
917. **Russell, A. D., and J. R. Furr.** 1996. Biocides: mechanisms of antifungal action and fungal resistance. Sci Prog **79:**27-48.
918. **Russell, A. D., and W. B. Hugo.** 1994. Antimicrobial activity and action of silver. Prog Med Chem **31:**351-70.
919. **Russell, A. D., and W. B. Hugo.** 1987. Chemical disinfectants. *In* A. H. Linton, W. B. Hugo and A. D. Russell (ed.), Disinfection in veterinary and farm animal practice. Blackwell Scientific Publications, Oxford, England.
920. **Russell, A. D., and G. McDonnell.** 2000. Concentration: a major factor in studying biocidal action. J Hosp Infect **44:**1-3.
921. **Russell, A. D., U. Tattawasart, J. Y. Maillard, and J. R. Furr.** 1998. Possible link between bacterial resistance and use of antibiotics and biocides. Antimicrob Agents Chemother **42:**2151.
922. **Rutala, W. A.** 1990. APIC guideline for selection and use of disinfectants. Am J Infect Control **18:**99-117.

923. **Rutala, W. A., S. L. Barbee, N. C. Aguiar, M. D. Sobsey, and D. J. Weber.** 2000. Antimicrobial activity of home disinfectants and natural products against potential human pathogens. Infect Control Hosp Epidemiol **21:**33-8.
924. **Rutala, W. A., E. P. Clontz, D. J. Weber, and K. K. Hoffmann.** 1991. Disinfection practices for endoscopes and other semicritical items. Infect Control Hosp Epidemiol **12:**282-8.
925. **Rutala, W. A., E. C. Cole, N. S. Wannamaker, and D. J. Weber.** 1991. Inactivation of *Mycobacterium tuberculosis* and *Mycobacterium bovis* by 14 hospital disinfectants. Am J Med **91:**267S-71S.
926. **Rutala, W. A., V. A. Kennedy, H. B. Loflin, and F. A. Sarubbi, Jr.** 1981. *Serratia marcescens* nosocomial infections of the urinary tract associated with urine measuring containers and urinometers. Am J Med **70:**659-63.
927. **Rutala, W. A., M. M. Stiegel, F. A. Sarubbi, and D. J. Weber.** 1997. Susceptibility of antibiotic-susceptible and antibiotic-resistant hospital bacteria to disinfectants. Infect Control Hosp Epidemiol **18:**417-21.
928. **Rutala, W. A., and D. J. Weber.** 1999. Disinfection of endoscopes: review of new chemical sterilants used for high-level disinfection. Infect Control Hosp Epidemiol **20:**69-76.
929. **Rutherford, K., J. Parkhill, J. Crook, T. Horsnell, P. Rice, M. A. Rajandream, and B. Barrell.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944-5.
930. **Ryu, H. J., W. J. Kim, C. H. Oh, and H. J. Song.** 2005. Iatrogenic *Mycobacterium abscessus* infection associated with acupuncture: clinical manifestations and its treatment. Int J Dermatol **44:**846-50.
931. **Saadat, S., and C. E. Ballou.** 1983. Pyruvylated glycolipids from *Mycobacterium smegmatis*. Structures of two oligosaccharide components. J Biol Chem **258:**1813-8.
932. **Saeki, A., N. Kido, T. Sugiyama, M. Ohta, T. Iwashita, K. Uchiya, and N. Kato.** 1993. Isolation of *rfb* gene clusters directing the synthesis of O polysaccharides consisting of mannose homopolymers and serological analysis of lipopolysaccharides. Microbiol Immunol **37:**601-6.
933. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487-91.
934. **Sakata, H., and S. Maruyama.** 1998. *Serratia marcescens* brain abscess in a newborn. Kansenshogaku Zasshi **72:**845-8.
935. **Salton, M. R.** 1968. Lytic agents, cell permeability, and monolayer penetrability. J Gen Physiol **52:**227-52.
936. **Sambrook, J.** 1989. Molecular cloning: a laboratory manual, vol. 2, Cold Spring Harbor, NY.
937. **Sanchez-Buenaventura, J., and L. Mustieles Blasco.** 1974. Epidemiologic study of the *Serratia* genus: *S. marcescens, S. liquefaciens,* and *S. rubidea*. Rev Sanid Hig Publica (Madr) **48:**291-315.
938. **Sanchez-Buenaventura, J., and M. L. Mustieles Blasco.** 1974. Rev Sanid Hig Publica **48:**291-315.
939. **Sander, P., E. De Rossi, B. Boddinghaus, R. Cantoni, M. Branzoni, E. C. Bottger, H. Takiff, R. Rodriquez, G. Lopez, and G. Riccardi.** 2000. Contribution of the multidrug efflux pump LfrA to innate mycobacterial drug resistance. FEMS Microbiol Lett **193:**19-23.
940. **Sanders, C. V., Jr., J. P. Luby, W. G. Johanson, Jr., J. A. Barnett, and J. P. Sanford.** 1970. *Serratia marcescens* infections from inhalation therapy medications: nosocomial outbreak. Ann Intern Med **73:**15-21.
941. **Sandossi, M., H. W. Rossmoore, and J. W. Wireman.** 1985. Observations of resistance and cross-resistance to formaldehyde and a formaldehyde condensate biocide in *Pseudomonas aeruginosa*. Int Biodeterior **21:**105–6.
942. **Sanguinetti, M., F. Ardito, E. Fiscarelli, M. La Sorda, P. D'Argenio, G. Ricciotti, and G. Fadda.** 2001. Fatal pulmonary infection due to multidrug-resistant *Mycobacterium abscessus* in a patient with cystic fibrosis. J Clin Microbiol **39:**816-9.
943. **Saraiva, M. A., C. M. Borges, and M. H. Florencio.** 2006. Reactions of a modified lysine with aldehydic and diketonic dicarbonyl compounds: an electrospray mass spectrometry structure/activity study. J Mass Spectrom **41:**216-28.
944. **Saraste, M.** 1999. Oxidative phosphorylation at the fin de siecle. Science **283:**1488-93.

945. **Sartor, C., V. Jacomo, C. Duvivier, H. Tissot-Dupont, R. Sambuc, and M. Drancourt.** 2000. Nosocomial *Serratia marcescens* infections associated with extrinsic contamination of a liquid nonmedicated soap. Infect Control Hosp Epidemiol **21:**196-9.
946. **Sasatsu, M., K. Shimizu, N. Noguchi, and M. Kono.** 1993. Triclosan-resistant *Staphylococcus aureus*. Lancet **341:**756.
947. **Sasatsu, M., Y. Shirai, M. Hase, N. Noguchi, M. Kono, H. Behr, J. Freney, and T. Arai.** 1995. The origin of the antiseptic-resistance gene *ebr* in *Staphylococcus aureus*. Microbios **84:**161-9.
948. **Sasnauskas, K., R. Jomantiene, A. Januska, E. Lebediene, J. Lebedys, and A. Janulaitis.** 1992. Cloning and analysis of a *Candida maltosa* gene which confers resistance to formaldehyde in *Saccharomyces cerevisiae*. Gene **122:**207-11.
949. **Sautter, R. L., L. H. Mattman, and R. C. Legaspi.** 1984. *Serratia marcescens* meningitis associated with a contaminated benzalkonium chloride solution. Infect Control **5:**223-5.
950. **Scavizzi, M. R.** 1972. Existence of 2 transferable tetracycline resistance factors. Ann Inst Pasteur **122:**3-17.
951. **Schaefler, S., J. Winter, A. Catelli, J. Greene, and B. Toharski.** 1971. Specific distribution of R factors in *Serratia marcescens* strains isolated from hospital infections. Appl Microbiol **22:**339-43.
952. **Schauder, S., and B. L. Bassler.** 2001. The languages of bacteria. Genes Dev **15:**1468-80.
953. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu Rev Microbiol **47:**597-626.
954. **Schlecht, S., and O. Westphal.** 1970. Investigations on the Typing of *Salmonella* R-forms. IV. Typing of *S. minnesota*-R-mutants by antibiotics. Zentralbl Bakteriol **213:**356-80.
955. **Schroder, O., and R. Wagner.** 2000. The bacterial DNA-binding protein H-NS represses ribosomal RNA transcription by trapping RNA polymerase in the initiation complex. J Mol Biol **298:**737-48.
956. **Schultze, W. D., and W. B. Brasso.** 1987. Characterization and identification of *Mycobacterium smegmatis* in bovine mastitis. Am J Vet Res **48:**739-42.
957. **Schulze-Robbecke, R.** 1993. Mycobacteria in the environment. Immun Infekt **21:**126-31.
958. **Schulze-Robbecke, R., and K. Buchholtz.** 1992. Heat susceptibility of aquatic mycobacteria. Appl Environ Microbiol **58:**1869-73.
959. **Schweizer, H. P.** 2003. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. Genet Mol Res **2:**48-62.
960. **Schweizer, H. P.** 1998. Intrinsic resistance to inhibitors of fatty acid biosynthesis in *Pseudomonas aeruginosa* is due to efflux: application of a novel technique for generation of unmarked chromosomal mutations for the study of efflux systems. Antimicrob Agents Chemother **42:**394-8.
961. **Schweizer, H. P.** 2001. Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiol Lett **202:**1-7.
962. **Schweizer, M., and U. Henning.** 1977. Action of a major outer cell envelope membrane protein in conjugation of *Escherichia coli* K-12. J Bacteriol **129:**1651-2.
963. **Schweizer, M., I. Hindennach, W. Garten, and U. Henning.** 1978. Major proteins of the *Escherichia coli* outer cell envelope membrane. Interaction of protein II with lipopolysaccharide. Eur J Biochem **82:**211-7.
964. **Scully, F. E., P. A. Hogg, G. Kennedy, C. Lewicki, A. M. Rule, and J. G. Soffriti.** 1999. Development of disinfection-resistant bacteria during wastewater treatment. Water Environ Res **71:**277.
965. **Seaman, P. F., D. Ochs, and M. J. Day.** 2007. Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother **59:**43-50.
966. **Seiler, D. A. L., and N. J. Russell.** 1991. Ethanol as a food preservative, p. 153-71. *In* G. W. Gould (ed.), Food preservatives. Blackie, Glasgow and London, UK.
967. **Seligman, M. L., and H. G. Mandel.** 1971. Inhibition of growth and RNA biosynthesis of *Bacillus cereus* by quinacrine. J Gen Microbiol **68:**135-48.
968. **Serruys-Schoutens, E., F. Rost, and G. Depre.** 1984. A nosocomial epidemic of *Serratia liquefaciens* urinary tract infection after cystometry. Eur J Clin Microbiol **3:**316-7.
969. **Sette, V.** 1824. Memoria storico naturale sull' l'arrossimento straodinario di alcune sostanze alimentos osservato nella provinicia di padova l'anno 1819. Venezia: Alvisopoli:63.

970. **Shackelford, J. C., G. W. Hanlon, and J. Y. Maillard.** 2006. Use of a new alginate film test to study the bactericidal efficacy of the high-level disinfectant *ortho*-phthalaldehyde. J Antimicrob Chemother **57:**335-8.
971. **Shahcheraghi, F., Y. Minato, J. Chen, T. Mizushima, W. Ogawa, T. Kuroda, and T. Tsuchiya.** 2007. Molecular cloning and characterization of a multidrug efflux pump, SmfY, from *Serratia marcescens*. Biol Pharm Bull **30:**798-800.
972. **Shao, J., B. Zhou, B. Chu, and Y. Yen.** 2006. Ribonucleotide reductase inhibitors and future drug design. Curr Cancer Drug Targets **6:**409-31.
973. **Sharma, V. K., and R. L. Zuerner.** 2004. Role of *hha* and *ler* in transcriptional regulation of the *esp* operon of enterohemorrhagic *Escherichia coli* O157:H7. J Bacteriol **186:**7290-301.
974. **Shepard, C. C.** 1957. Growth characteristics in HeLa cells of the rapidly growing acid fast bacteria, *Mycobacterium fortuitum*, *Mycobacterium phlei,* and *Mycobacterium smegmatis*. J Bacteriol **73:**722-6.
975. **Shin, M., M. Song, J. H. Rhee, Y. Hong, Y. J. Kim, Y. J. Seok, K. S. Ha, S. H. Jung, and H. E. Choy.** 2005. DNA looping-mediated repression by histone-like protein H-NS: specific requirement of Esigma70 as a cofactor for looping. Genes Dev **19:**2388-98.
976. **Shinnick, T. M., and R. C. Good.** 1994. Mycobacterial taxonomy. Eur J Clin Microbiol Infect Dis **13:** 884–90.
977. **Shockman, G. D., L. Daneo-Moore, and M. L. Higgins.** 1974. Problems of cell wall and membrane growth, enlargement, and division. Ann N Y Acad Sci **235:**161-97.
978. **Sibley, L. D., S. W. Hunter, P. J. Brennan, and J. L. Krahenbuhl.** 1988. Mycobacterial lipoarabinomannan inhibits gamma interferon-mediated activation of macrophages. Infect Immun **56:**1232-6.
979. **Sidhu, M. S., E. Heir, T. Leegaard, K. Wiger, and A. Holck.** 2002. Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon *Tn*552 among clinical staphylococci. Antimicrob Agents Chemother **46:**2797-803.
980. **Sidhu, M. S., E. Heir, H. Sorum, and A. Holck.** 2001. Genetic linkage between resistance to quaternary ammonium compounds and beta-lactam antibiotics in food-related *Staphylococcus* spp. Microb Drug Resist **7:**363-71.
981. **Sigler, P. B., Z. Xu, H. S. Rye, S. G. Burston, W. A. Fenton, and A. L. Horwich.** 1998. Structure and function in GroEL-mediated protein folding. Annu Rev Biochem **67:**581-608.
982. **Silcox, V. A., R. C. Good, and M. M. Floyd.** 1981. Identification of clinically significant *Mycobacterium fortuitum* complex isolates. J Clin Microbiol **14:**686-91.
983. **Silphaduang, U., M. Mascarenhas, M. Karmali, and B. K. Coombes.** 2007. Repression of intracellular virulence factors in *Salmonella* by the Hha and YdgT nucleoid-associated proteins. J Bacteriol **189:**3669-73.
984. **Silver, S.** 1967. Acridine sensitivity of bacteriophage T2: a virus gene affecting cell permeability. J Mol Biol **29:**191-202.
985. **Silver, S., and T. K. Misra.** 1988. Plasmid-mediated heavy metal resistances. Annu Rev Microbiol **42:**717-43.
986. **Simons, C., S. E. Walsh, J. Y. Maillard, and A. D. Russell.** 2000. A note: *ortho*-phthalaldehyde: proposed mechanism of action of a new antimicrobial agent. Lett Appl Microbiol **31:**299-302.
987. **Skiest, D. J., and M. E. Levi.** 1998. Catheter-related bacteremia due to *Mycobacterium smegmatis*. South Med J **91:**36-7.
988. **Skurray, R. A., D. A. Rouch, B. R. Lyon, M. T. Gillespie, J. M. Tennent, M. E. Byrne, L. J. Messerotti, and J. W. May.** 1988. Multiresistant *Staphylococcus aureus*: genetics and evolution of epidemic Australian strains. J Antimicrob Chemother **21 (Suppl C):**19-39.
989. **Slater, H., M. Crow, L. Everson, and G. P. Salmond.** 2003. Phosphate availability regulates biosynthesis of two antibiotics, prodigiosin and carbapenem, in *Serratia* via both quorum-sensing-dependent and -independent pathways. Mol Microbiol **47:**303-20.
990. **Slayden, R. A., R. E. Lee, and C. E. Barry, 3rd.** 2000. Isoniazid affects multiple components of the type II fatty acid synthase system of *Mycobacterium tuberculosis*. Mol Microbiol **38:**514-25.
991. **Sleigh, J. D.** 1983. Antibiotic resistance in *Serratia marcescens*. Br Med J (Clin Res Ed) **287:**1651-2.
992. **Smith, C. R.** 1968. Microbactericidal agents. *In* C. A. Lawrence and S. S. Block (ed.), Disinfection, sterilization, and preservation. Lea & Febiger, Philadelphia.

993. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol Microbiol **4:**1911-9.
994. **Sofos, J. N., and F. F. Busta.** 1999. Chemical food preservatives, p. 485–541. *In* A. D. Russell, W. B. Hugo and G. A. J. Ayliffe (ed.), Principles and practice of disinfection and preservation, 3rd ed. Blackwell Science, Oxford, UK.
995. **Sogaard-Andersen, L., J. Martinussen, N. E. Mollegaard, S. R. Douthwaite, and P. Valentin-Hansen.** 1990. The CytR repressor antagonizes cyclic AMP-cyclic AMP receptor protein activation of the *deoCp2* promoter of *Escherichia coli* K-12. J Bacteriol **172:**5706-13.
996. **Sonntag, I., H. Schwarz, Y. Hirota, and U. Henning.** 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins. J Bacteriol **136:**280-5.
997. **Soo, P. C., Y. T. Horng, M. J. Lai, J. R. Wei, S. C. Hsieh, Y. L. Chang, Y. H. Tsai, and H. C. Lai.** 2007. Pirin regulates pyruvate catabolism by interacting with the pyruvate dehydrogenase E1 subunit and modulating pyruvate dehydrogenase activity. J Bacteriol **189:**109-18.
998. **Soto, G. E., and S. J. Hultgren.** 1999. Bacterial adhesins: common themes and variations in architecture and assembly. J Bacteriol **181:**1059-71.
999. **Spaulding, E. H.** 1964 Alcohol as a surgical disinfectant. Assoc Operating Room Nurses J **2:**67-71.
1000. **Spaulding, E. H., K. R. Cundy, and F. J. Turner.** 1977. Chemical disinfection of medical and surgical materials, p. 654-84. *In* S. S. Block (ed.), Disinfection, sterilization and preservation vol. 2. Lea & Febiger, Philadelphia.
1001. **Sreevatsan, S., K. E. Stockbauer, X. Pan, B. N. Kreiswirth, S. L. Moghazeh, W. R. Jacobs, Jr., A. Telenti, and J. M. Musser.** 1997. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. Antimicrob Agents Chemother **41:**1677-81.
1002. **Stackebrandt, E., F. A. Rainey, and N. L. Ward-Rainey.** 1997. Proposal for a new hierarchic classification system, *Actinobacteria classis* nov. Int J Syst Bacteriol **47:** 479-91.
1003. **Stamminger, G., M. Brendel, and R. W. Kaplan.** 1973. Co-transduction mapping in *Serratia marcescens*. Z Allg Mikrobiol **13:**59-70.
1004. **Stanford, J. L., S. R. Pattyn, F. Portaels, and W. J. Gunthorpe.** 1972. Studies on *Mycobacterium chelonei*. J Med Microbiol **5:**177-82.
1005. **Stanley, P.** 1998. Destruction of glutaraldehyde-resistant mycobacterium by a peroxygen disinfectant. Am J Infect Control **26:**185.
1006. **Stapleton, P. D., K. P. Shannon, and G. L. French.** 1999. Carbapenem resistance in *Escherichia coli* associated with plasmid-determined CMY-4 beta-lactamase production and loss of an outer membrane protein. Antimicrob Agents Chemother **43:**1206-10.
1007. **Steiger, H., U. Muller, and G. Bauer.** 1972. Non-receptivity for kappa phage of kappa-lysogenic *Serratia* and reactions to superinfection of receptive cells with a mutant prophage. Mol Gen Genet **114:**358-67.
1008. **Steingrube, V. A., J. L. Gibson, B. A. Brown, Y. Zhang, R. W. Wilson, M. Rajagopalan, and R. J. Wallace, Jr.** 1995. PCR amplification and restriction endonuclease analysis of a 65-kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. J Clin Microbiol **33:**149-53.
1009. **Steinhaus, E. A.** 1959. *Serratia marcescens* Bizio as an insect pathogen. Hilgardia. **28:**351-80
1010. **Steinhaus, E. A.** 1951. Report on diagnoses of diseased insects 1944–1950. Hilgardia **20:**629–78.
1011. **Steinhaus, E. A.** 1941. A study of the bacteria associated with thirty species of insects. J Bacteriol **42:**757-90.
1012. **Steinmetz, M., D. Le Coq, S. Aymerich, G. Gonzy-Treboul, and P. Gay.** 1985. The DNA sequence of the gene for the secreted *Bacillus subtilis* enzyme levansucrase and its genetic control sites. Mol Gen Genet **200:**220-8.
1013. **Stenderup, A., O. Faergeman, and M. Ingerslev.** 1966. *Serratia marcescens* infections in premature infants. Acta Pathol Microbiol Scand **68:**157-60.
1014. **Stephan, J., C. Mailaender, G. Etienne, M. Daffe, and M. Niederweis.** 2004. Multidrug resistance of a porin deletion mutant of *Mycobacterium smegmatis*. Antimicrob Agents Chemother **48:**4163-70.

1015. **Stephen, M., and M. K. Lalitha.** 1993. An outbreak of *Serratia marcescens* infection among obstetric patients. Indian J Med Res **97**:202-5.
1016. **Stephens, J. M.** 1963. J Insect Pathol **5**:61-5.
1017. **Stickler, D. J.** 2004. Bacterial resistance: Intrinsic resistance of Gram-negative bacteria, p. 154-69. *In* A. P. Fraise, P. A. Lambert and J-Y. Maillard (ed.), Principles and practice of disinfectation, preservation and sterilization, 4th ed. Blackwell Publishing, Oxford, England.
1018. **Stickler, D. J.** 1974. Chlorhexidine resistance in *Proteus mirabilis*. J Clin Pathol **27**:284-7.
1019. **Stickler, D. J., and B. Thomas.** 1980. Antiseptic and antibiotic resistance in Gram-negative bacteria causing urinary tract infection. J Clin Pathol **33**:288-96.
1020. **Stickler, D. J., and B. Thomas.** 1976. Sensitivity of *Providence* to antiseptics and disinfectants. J Clin Pathol **29**:815-23.
1021. **Stickler, D. J., B. Thomas, and J. C. Chawla.** 1981. Antiseptic and antibiotic resistance in gram-negative bacteria causing urinary tract infection in spinal cord injured patients. Paraplegia **19**:50-8.
1022. **Stock, I., T. Grueger, and B. Wiedemann.** 2003. Natural antibiotic susceptibility of strains of *Serratia marcescens* and the *S. liquefaciens* complex: *S. liquefaciens* sensu stricto, *S. proteamaculans* and *S. grimesii*. Int J Antimicrob Agents **22**:35-47.
1023. **Stoeckle, M. Y., L. Guan, N. Riegler, I. Weitzman, B. Kreiswirth, J. Kornblum, F. Laraque, and L. W. Riley.** 1993. Catalase-peroxidase gene sequences in isoniazid-sensitive and -resistant strains of *Mycobacterium tuberculosis* from New York City. J Infect Dis **168**:1063-5.
1024. **Stubbe, J., and W. A. van Der Donk.** 1998. Protein radicals in enzyme catalysis. Chem Rev **98**:705-62.
1025. **Sturme, M. H., M. Kleerebezem, J. Nakayama, A. D. Akkermans, E. E. Vaugha, and W. M. de Vos.** 2002. Cell to cell communication by autoinducing peptides in gram-positive bacteria. Antonie Van Leeuwenhoek **81**:233-43.
1026. **Su, L. H., J. T. Ou, H. S. Leu, P. C. Chiang, Y. P. Chiu, J. H. Chia, A. J. Kuo, C. H. Chiu, C. Chu, T. L. Wu, and *et al.*** 2003. Extended epidemic of nosocomial urinary tract infections caused by *Serratia marcescens*. J Clin Microbiol **41**:4726-32.
1027. **Sugawara, E., and H. Nikaido.** 1994. OmpA protein of *Escherichia coli* outer membrane occurs in open and closed channel forms. J Biol Chem **269**:17981-7.
1028. **Sukupolvi, S., M. Vaara, I. M. Helander, P. Viljanen, and P. H. Makela.** 1984. New *Salmonella typhimurium* mutants with altered outer membrane permeability. J Bacteriol **159**:704-12.
1029. **Sulavik, M. C., C. Houseweart, C. Cramer, N. Jiwani, N. Murgolo, J. Greene, B. DiDomenico, K. J. Shaw, G. H. Miller, R. Hare, and G. Shimer.** 2001. Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother **45**:1126-36.
1030. **Suller, M. T., and A. D. Russell.** 1999. Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. J Hosp Infect **43**:281-91.
1031. **Suller, M. T., and A. D. Russell.** 2000. Triclosan and antibiotic resistance in *Staphylococcus aureus*. J Antimicrob Chemother **46**:11-8.
1032. **Sun, L., and J. A. Fuchs.** 1992. *Escherichia coli* ribonucleotide reductase expression is cell cycle regulated. Mol Biol Cell **3**:1095-105.
1033. **Surolia, N., and A. Surolia.** 2001. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *Plasmodium falciparum*. Nat Med **7**:167-73.
1034. **Swarup, S., R. DeFeyter, R. H. Brlansky, and D. W. Gabriel.** 1991. pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *Xanthomonas campestris* to elicit cankerlike lesions on citrus. Phytopathology **81**:802–9.
1035. **Swenson, J. M., C. Thornsberry, and V. A. Silcox.** 1982. Rapidly growing mycobacteria: testing of susceptibility to 34 antimicrobial agents by broth microdilution. Antimicrob Agents Chemother **22**:186-92.
1036. **Swenson, J. M., R. J. Wallace, Jr., V. A. Silcox, and C. Thornsberry.** 1985. Antimicrobial susceptibility of five subgroups of *Mycobacterium fortuitum* and *Mycobacterium chelonae*. Antimicrob Agents Chemother **28**:807-11.
1037. **Swift, S., A. V. Karlyshev, L. Fish, E. L. Durant, M. K. Winson, S. R. Chhabra, P. Williams, S. Macintyre, and G. S. Stewart.** 1997. Quorum sensing in *Aeromonas hydrophila* and

Aeromonas salmonicida: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. J Bacteriol **179:**5271-81.

1038. **Sykes, G.** 1965. Disinfection and sterilization, 2nd ed. E. & F.N. Spon., London.

1039. **Taber, H. W., J. P. Mueller, P. F. Miller, and A. S. Arrow.** 1987. Bacterial uptake of aminoglycoside antibiotics. Microbiol Rev **51:**439-57.

1040. **Takano, E., T. Nihira, Y. Hara, J. J. Jones, C. J. Gershater, Y. Yamada, and M. Bibb.** 2000. Purification and structural determination of SCB1, a gamma-butyrolactone that elicits antibiotic production in *Streptomyces coelicolor* A3(2). J Biol Chem **275:**11010-6.

1041. **Takasaki, A., T. Hashida, S. Fujiwara, K.-I. Kato, and T. Nishihara.** 1994. Bactericidal action of a quaternary ammonium disinfectant, didecyldimethyl ammonium chloride, against *Staphylococcus aureus*. Jap J Toxicol Environ Health **40:**344–50.

1042. **Takasaki, A., T. Hashida, K.-I. Kato, T. Moriyama, and T. Nishihara.** 1994. Action of a quaternary ammonium disinfectant on cell membrane of *Staphylococcus aureus*. Jap J Toxicol Environ Health **40:**520–6.

1043. **Takehiko, M., S. Joshi, M. Gomez-Escalada, and P. H. Schweizer.** 2007. Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. J Bacteriol **189:**7600-9.

1044. **Takeo, S., A. Kokaze, C. S. Ng, D. Mizuchi, J. I. Watanabe, K. Tanabe, S. Kojima, and K. Kita.** 2000. Succinate dehydrogenase in *Plasmodium falciparum* mitochondria: molecular characterization of the SDHA and SDHB genes for the catalytic subunits, the flavoprotein (Fp) and iron-sulfur (Ip) subunits. Mol Biochem Parasitol **107:**191-205.

1045. **Takiff, H. E., M. Cimino, M. C. Musso, T. Weisbrod, R. Martinez, M. B. Delgado, L. Salazar, B. R. Bloom, and W. R. Jacobs, Jr.** 1996. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. Proc Natl Acad Sci USA **93:**362-6.

1046. **Talaat, A. M., M. Trucksis, A. S. Kane, and R. Reimschuessel.** 1999. Pathogenicity of *Mycobacterium fortuitum* and *Mycobacterium smegmatis* to goldfish, *Carassius auratus*. Vet Microbiol **66:**151-64.

1047. **Tamaki, S., and M. Matsuhashi.** 1973. Increase in sensitivity to antibiotics and lysozyme on deletion of lipopolysaccharides in *Escherichia coli* strains. J Bacteriol **114:**453-4.

1048. **Tattawasart, U., A. C. Hann, J. Y. Maillard, J. R. Furr, and A. D. Russell.** 2000. Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*. J Antimicrob Chemother **45:**145-52.

1049. **Tattawasart, U., J. Y. Maillard, J. R. Furr, and A. D. Russell.** 1999. Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in *Pseudomonas stutzeri* and changes in antibiotic susceptibility. J Hosp Infect **42:**219-29.

1050. **Tattawasart, U., J. Y. Maillard, J. R. Furr, and A. D. Russell.** 2000. Outer membrane changes in *Pseudomonas stutzeri* resistant to chlorhexidine diacetate and cetylpyridinium chloride. Int J Antimicrob Agents **16:**233-8.

1051. **Telenti, A., F. Marchesi, M. Balz, F. Bally, E. C. Bottger, and T. Bodmer.** 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol **31:**175-8.

1052. **Telenti, A., W. J. Philipp, S. Sreevatsan, C. Bernasconi, K. E. Stockbauer, B. Wieles, J. M. Musser, and W. R. Jacobs, Jr.** 1997. The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nat Med **3:**567-70.

1053. **Telford, J. R., and K. N. Raymond.** 1996. Siderophores. Compr Supramol Chem **1:**245–66.

1054. **Tennent, J. M., B. R. Lyon, M. Midgley, I. G. Jones, A. S. Purewal, and R. A. Skurray.** 1989. Physical and biochemical characterization of the *qacA* gene encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. J Gen Microbiol **135:**1-10.

1055. **Teplitski, M., J. B. Robinson, and W. D. Bauer.** 2000. Plants secrete substances that mimic bacterial *N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. Mol Plant Microbe Interact **13:**637-48.

1056. **Thoen, C. O., A. G. Karlson, and E. M. Himes.** 1981. Mycobacterial infections in animals. Rev Infect Dis **3:**960-72.

1057. **Thomson, J. A.** 1977. *E. coli* phosphofructokinase synthesized *in vitro* from a ColE1 hybrid plasmid. Gene **1:**347-56.

1058. **Thomson, J. R., N. Mollison, and K. P. Matthews.** 1988. An investigation of mastitis due to *S. agalactiae, S. uberis and M. smegmatis* in a dairy herd. Vet Rec **122:**271-4.
1059. **Thomson, N. R., M. A. Crow, S. J. McGowan, A. Cox, and G. P. Salmond.** 2000. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in *Serratia* is under quorum sensing control. Mol Microbiol **36:**539-56.
1060. **Thornalley, P. J.** 2003. Glyoxalase I--structure, function and a critical role in the enzymatic defence against glycation. Biochem Soc Trans **31:**1343-8.
1061. **Thurman, R. B., and C. P. Gerba.** 1988. Molecular mechanisms of viral inactivation by water disinfectants. Adv Appl Microbiol **33:**75-105.
1062. **Thurman, R. B., and C. P. Gerba.** 1989. The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. Crit Rev Environ Contr **18:**295–315.
1063. **Tilley, F. W., A. D. MacDonald, and J. M. Schaffer.** 1931. Germicidal efficiency of o-phenylphenol against *Mycobacterium tuberculosis*. J Agric Res **42:**653.
1064. **Tortoli, E.** 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev **16:**319-54.
1065. **Tortosa, P., and D. Dubnau.** 1999. Competence for transformation: a matter of taste. Curr Opin Microbiol **2:**588-92.
1066. **Tosi, L. R., and S. M. Beverley.** 2000. *cis* and *trans* factors affecting Mos1 *mariner* evolution and transposition *in vitro*, and its potential for functional genomics. Nucleic Acids Res **28:**784-90.
1067. **Toth-Prestia, C., and I. N. Hirshfield.** 1988. Isolation of plasmid-harboring *Serratia plymuthica* from facultative gut microflora of the tobacco hornworm, *Manduca sexta*. Appl Environ Microbiol **54:**1855-7.
1068. **Townsend, D. E., L. Greed, N. Ashdown, and W. B. Grubb.** 1983. Plasmid-mediated resistance to quaternary ammonium compounds in methicillin-resistant *Staphylococcus aureus*. Med J Aust **2:**310.
1069. **Townsend, D. E., W. B. Grubb, and N. Ashdown.** 1983. Gentamicin resistance in methicillin-resistant *Staphylococcus aureus*. Pathology **15:**169-74.
1070. **Traore, O., F. A. Allaert, S. Fournet-Fayard, J. L. Verriere, and H. Laveran.** 2000. Comparison of in-vivo antibacterial activity of two skin disinfection procedures for insertion of peripheral catheters: povidone iodine versus chlorhexidine. J Hosp Infect **44:**147-50.
1071. **Traub, W. H., and D. Bauer.** 1987. Outer membrane protein alterations in *Serratia marcescens* resistant against aminoglycoside and beta-lactam antibiotics. Chemotherapy **33:**172-6.
1072. **Trevisan, V.** 1889. I. Generi e le Specie delle Batteriacee, Zanaboni and Gabuzzi, Milano.
1073. **Trevor, J. T.** 1987. Silver resistance and accumulation in bacteria. Enzyme Microb Technol **9:**331-3.
1074. **Trias, J., and R. Benz.** 1994. Permeability of the cell wall of *Mycobacterium smegmatis*. Mol Microbiol **14:**283-90.
1075. **Tseng, T. T., K. S. Gratwick, J. Kollman, D. Park, D. H. Nies, A. Goffeau, and M. H. Saier, Jr.** 1999. The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. J Mol Microbiol Biotechnol **1:**107-25.
1076. **Tuomanen, E., R. Cozens, W. Tosch, O. Zak, and A. Tomasz.** 1986. The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. J Gen Microbiol **132:**1297-304.
1077. **Tuomanen, E., and J. Schwartz.** 1987. Penicillin-binding protein 7 and its relationship to lysis of nongrowing *Escherichia coli*. J Bacteriol **169:**4912-5.
1078. **Turner, N. A., J. Harris, A. D. Russell, and D. Lloyd.** 2000. Microbial differentiation and changes in susceptibility to antimicrobial agents. J Appl Microbiol **89:**751-9.
1079. **Turner, N. A., A. D. Russell, J. R. Furr, and D. Lloyd.** 1999. *Acanthamoeba* spp., antimicrobial agents and contact lenses. Sci Prog **82:**1-8.
1080. **Tyler, R., G. A. Ayliffe, and C. Bradley.** 1990. Virucidal activity of disinfectants: studies with the poliovirus. J Hosp Infect **15:**339-45.
1081. **Uotila, L., and M. Koivusalo.** 1989. Glutathione-dependent oxireductases: formaldehyde dehydrogenase, p. 517–50. *In* D. Dolphin, R. Poulson and O. Abramovic (ed.), Coenzymes and cofactors. Wiley, New York.

1082. **Uyeda, M., T. Shiozaki, T. Enokida, K. Watanabe, K. Komiya, K. Yokomizo, and K. Suzuki.** 1996. Microbial degradation of chlorhexidine by clinical isolates. Jpn J Toxicol Environ Health **42:**312-8.
1083. **Vaara, M.** 1992. Agents that increase the permeability of the outer membrane. Microbiol Rev **56:**395-411.
1084. **Vaara, M., and T. Vaara.** 1983. Polycations sensitize enteric bacteria to antibiotics. Antimicrob Agents Chemother **24:**107-13.
1085. **Valencia, I. C., E. Weiss, E. Sukenik, and F. A. Kerdel.** 1999. Disseminated cutaneous *Mycobacterium chelonae* infection after injection of bovine embryonic cells. Int J Dermatol **38:**770-3.
1086. **van Bueren, J., D. P. Larkin, and R. A. Simpson.** 1994. Inactivation of human immunodeficiency virus type 1 by alcohols. J Hosp Infect **28:**137-48.
1087. **van de Klundert, J. A., M. H. van Gestel, G. Meerdink, and S. de Marie.** 1988. Emergence of bacterial resistance to cefamandole *in vivo* due to outer membrane protein deficiency. Eur J Clin Microbiol Infect Dis **7:**776-8.
1088. **van Houdt, R., M. Givskov, and C. W. Michiels.** 2007. Quorum sensing in *Serratia*. FEMS Microbiol Rev **31:**407-24.
1089. **van Klingeren, B., and W. Pullen.** 1987. Comparative testing of disinfectants against *Mycobacterium tuberculosis* and *Mycobacterium terrae* in a quantitative suspension test. J Hosp Infect **10:**292-8.
1090. **van Klingeren, B., and W. Pullen.** 1993. Glutaraldehyde resistant mycobacteria from endoscope washers. J Hosp Infect **25:**147-9.
1091. **van Veen, H. W., and W. N. Konings.** 1998. The ABC family of multidrug transporters in microorganisms. Biochim Biophys Acta **1365:**31-6.
1092. **Vigeant, P., V. G. Loo, C. Bertrand, C. Dixon, R. Hollis, M. A. Pfaller, A. P. McLean, D. J. Briedis, T. M. Perl, and H. G. Robson.** 1998. An outbreak of *Serratia marcescens* infections related to contaminated chlorhexidine. Infect Control Hosp Epidemiol **19:**791-4.
1093. **Vigneulle, M., and F. B. Laurencin.** 1995. *Serratia liquefaciens*: a case report in turbot (*Scophthalmus maximus*) cultured in floating cages in France. Source Aquaculture **132:**121-24.
1094. **Viljanen, P., and M. Vaara.** 1984. Susceptibility of gram-negative bacteria to polymyxin B nonapeptide. Antimicrob Agents Chemother **25:**701-5.
1095. **Villalain, J., C. R. Mateo, F. J. Aranda, S. Shapiro, and V. Micol.** 2001. Membranotropic effects of the antibacterial agent Triclosan. Arch Biochem Biophys **390:**128-36.
1096. **Villanueva, A., R. V. Calderon, B. A. Vargas, F. Ruiz, S. Aguero, Y. Zhang, B. A. Brown, and R. J. Wallace, Jr.** 1997. Report on an outbreak of postinjection abscesses due to *Mycobacterium abscessus*, including management with surgery and clarithromycin therapy and comparison of strains by random amplified polymorphic DNA polymerase chain reaction. Clin Infect Dis **24:**1147-53.
1097. **Viveiros, M., C. Leandro, and L. Amaral.** 2003. Mycobacterial efflux pumps and chemotherapeutic implications. Int J Antimicrob Agents **22:**274-8.
1098. **Vonmoos, S., P. Leuenberger, V. Beer, and R. de Haller.** 1986. Pleuropulmonary infection caused by *Mycobacterium smegmatis*. Case description and literature review. Schweiz Med Wochenschr **116:**1852-6.
1099. **Waaler, S. M., G. Rolla, K. K. Skjorland, and B. Ogaard.** 1993. Effects of oral rinsing with triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study. Scand J Dent Res **101:**192-5.
1100. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol Rev **48:**60-93.
1101. **Wallace, R. J., Jr, J. Glassroth, D.E. Griffith, K.N. Olivier, J.L. Cook and F. Gordin. American Thoracic Society.** 1997. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. Am J Resp Crit Care Med **156:**S1-S25.
1102. **Wallace, R. J., Jr.** 1989. The clinical presentation, diagnosis, and therapy of cutaneous and pulmonary infections due to the rapidly growing mycobacteria, *M. fortuitum* and *M. chelonae*. Clin Chest Med **10:**419-29.

1103. **Wallace, R. J., Jr., B. A. Brown-Elliott, L. Hall, G. Roberts, R. W. Wilson, L. B. Mann, C. J. Crist, S. H. Chiu, R. Dunlap, M. J. Garcia, J. T. Bagwell, and K. C. Jost, Jr.** 2002. Clinical and laboratory features of *Mycobacterium mageritense*. J Clin Microbiol **40:**2930-5.
1104. **Wallace, R. J., Jr., B. A. Brown-Elliott, S. C. Ward, C. J. Crist, L. B. Mann, and R. W. Wilson.** 2001. Activities of linezolid against rapidly growing mycobacteria. Antimicrob Agents Chemother **45:**764-7.
1105. **Wallace, R. J., Jr., and B. A. Brown.** 1999. *Mycobacterium fortuitum, chelonae, abscessus*, p. 372–9. *In* D. Schlossberg (ed.), Tuberculosis and nontuberculous mycobacterial infections. The W. B. Saunders Co, Philadelphia.
1106. **Wallace, R. J., Jr., B. A. Brown, and D. E. Griffith.** 1998. Nosocomial outbreaks/pseudo-outbreaks caused by nontuberculous mycobacteria. Annu Rev Microbiol **52:**453-90.
1107. **Wallace, R. J., Jr., B. A. Brown, and G. O. Onyi.** 1992. Skin, soft tissue, and bone infections due to *Mycobacterium chelonae*: importance of prior corticosteroid therapy, frequency of disseminated infections, and resistance to oral antimicrobials other than clarithromycin. J Infect Dis **166:**405-12.
1108. **Wallace, R. J., Jr., B. A. Brown, and G. O. Onyi.** 1991. Susceptibilities of *Mycobacterium fortuitum* biovar. *fortuitum* and the two subgroups of *Mycobacterium chelonae* to imipenem, cefmetazole, cefoxitin, and amoxicillin-clavulanic acid. Antimicrob Agents Chemother **35:**773-5.
1109. **Wallace, R. J., Jr., J. R. Dalovisio, and G. A. Pankey.** 1979. Disk diffusion testing of susceptibility of *Mycobacterium fortuitum* and *Mycobacterium chelonei* to antibacterial agents. Antimicrob Agents Chemother **16:**611-4.
1110. **Wallace, R. J., Jr., J. M. Musser, S. I. Hull, V. A. Silcox, L. C. Steele, G. D. Forrester, A. Labidi, and R. K. Selander.** 1989. Diversity and sources of rapidly growing mycobacteria associated with infections following cardiac surgery. J Infect Dis **159:**708-16.
1111. **Wallace, R. J., Jr., D. R. Nash, M. Tsukamura, Z. M. Blacklock, and V. A. Silcox.** 1988. Human disease due to *Mycobacterium smegmatis*. J Infect Dis **158:**52-9.
1112. **Wallace, R. J., Jr., V. A. Silcox, M. Tsukamura, B. A. Brown, J. O. Kilburn, W. R. Butler, and G. Onyi.** 1993. Clinical significance, biochemical features, and susceptibility patterns of sporadic isolates of the *Mycobacterium chelonae*-like organism. J Clin Microbiol **31:**3231-9.
1113. **Wallace, R. J., Jr., L. C. Steele, A. Labidi, and V. A. Silcox.** 1989. Heterogeneity among isolates of rapidly growing mycobacteria responsible for infections following augmentation mammaplasty despite case clustering in Texas and other southern coastal states. J Infect Dis **160:**281-8.
1114. **Wallace, R. J., Jr., J. M. Swenson, V. A. Silcox, and M. G. Bullen.** 1985. Treatment of nonpulmonary infections due to *Mycobacterium fortuitum* and *Mycobacterium chelonei* on the basis of *in vitro* susceptibilities. J Infect Dis **152:**500-14.
1115. **Wallace, R. J., Jr., J. M. Swenson, V. A. Silcox, R. C. Good, J. A. Tschen, and M. S. Stone.** 1983. Spectrum of disease due to rapidly growing mycobacteria. Rev Infect Dis **5:**657-79.
1116. **Wallhäusser, K. H.** 1984. Antimicrobial preservatives used by the cosmetic industry, p. 605–745. *In* J. J. Kabara (ed.), Cosmetic and drug preservation: Principles and practice. Marcel Dekker. Sons, New York.
1117. **Walsh, P. S., D. A. Metzger, and R. Higuchi.** 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques **10:**506-13.
1118. **Walsh, S., J. Y. Maillard, and A. D. Russell.** 1997. Presented at the Society for Applied Microbiology Autumn Meeting.
1119. **Walsh, S. E., J. Y. Maillard, and A. D. Russell.** 1999. *Ortho*-phthalaldehyde: a possible alternative to glutaraldehyde for high level disinfection. J Appl Microbiol **86:**1039-46.
1120. **Walsh, S. E., J. Y. Maillard, A. D. Russell, and A. C. Hann.** 2001. Possible mechanisms for the relative efficacies of *ortho*-phthalaldehyde and glutaraldehyde against glutaraldehyde-resistant *Mycobacterium chelonae*. J Appl Microbiol **91:**80-92.
1121. **Walsh, S. E., J. Y. Maillard, C. Simons, and A. D. Russell.** 1999. Studies on the mechanisms of the antibacterial action of *ortho*-phthalaldehyde. J Appl Microbiol **87:**702-10.
1122. **Walter, S.** 2002. Structure and function of the GroE chaperone. Cell Mol Life Sci **59:**1589-97.
1123. **Wang, J. C.** 1991. DNA topoisomerases: why so many? J Biol Chem **266:**6659-62.
1124. **Wang, J. H., and A. T. Matheson.** 1967. The interaction of *Escherichia coli* ribosomes with p-chloromercuribenzoate. Biochim Biophys Acta **138:**296-306.

1125. **Wang, Y.** 2002. The function of OmpA in *Escherichia coli*. Biochem Biophys Res Commun **292:**396-401.
1126. **Wasem, C. F., C. M. McCarthy, and L. W. Murray.** 1991. Multilocus enzyme electrophoresis analysis of the *Mycobacterium avium* complex and other mycobacteria. J Clin Microbiol **29:**264-71.
1127. **Watanabe, M., S. Iyobe, M. Inoue, and S. Mitsuhashi.** 1991. Transferable imipenem resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother **35:**147-51.
1128. **Watson, D. W., and J. J. Petersen.** 1991. Infectivity of *Serratia marcescens* (*Eubacteriales: Enterobacteriaceae*) in *Stomoxys calcitrans* (Diptera: *Muscidae*). J Med Entomol **28:**190-2.
1129. **Wayne, L. G., and H. A. Sramek.** 1992. Agents of newly recognized or infrequently encountered mycobacterial diseases. Clin Microbiol Rev **5:**1-25.
1130. **Weavers, L. K., and G. B. Wickramanayake.** 2001. Disinfection and sterilization using ozone, p. 205-14. *In* S. S. Block (ed.), Disinfection, sterilization and preservation, 5th ed. Lippincott Williams & Wilkins, Philadelphia, USA.
1131. **Weber, G.** 1983. Biochemical strategy of cancer cells and the design of chemotherapy: G. H. A. Clowes Memorial Lecture. Cancer Res **43:**3466-92.
1132. **Weindorf, H., H. Schmidt, and H. H. Martin.** 1998. Contribution of overproduced chromosomal beta-lactamase and defective outer membrane porins to resistance to extended-spectrum beta-lactam antibiotics in *Serratia marcescens*. J Antimicrob Chemother **41:**189-95.
1133. **Weiner, J. H., R. Cammack, S. T. Cole, C. Condon, N. Honore, B. D. Lemire, and G. Shaw.** 1986. A mutant of *Escherichia coli* fumarate reductase decoupled from electron transport. Proc Natl Acad Sci USA **83:**2056-60.
1134. **Weiser, J. N., and E. C. Gotschlich.** 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect Immun **59:**2252-8.
1135. **Weiss, J., S. Beckerdite-Quagliata, and P. Elsbach.** 1980. Resistance of gram-negative bacteria to purified bactericidal leukocyte proteins: relation to binding and bacterial lipopolysaccharide structure. J Clin Invest **65:**619-28.
1136. **Weissman, J. S., H. S. Rye, W. A. Fenton, J. M. Beechem, and A. L. Horwich.** 1996. Characterization of the active intermediate of a GroEL-GroES-mediated protein folding reaction. Cell **84:**481-90.
1137. **Wendler, W. M., E. Kremmer, R. Forster, and E. L. Winnacker.** 1997. Identification of pirin, a novel highly conserved nuclear protein. J Biol Chem **272:**8482-9.
1138. **Wenger, J. D., J. S. Spika, R. W. Smithwick, V. Pryor, D. W. Dodson, G. A. Carden, and K. C. Klontz.** 1990. Outbreak of *Mycobacterium chelonae* infection associated with use of jet injectors. JAMA **264:**373-6.
1139. **Wheat, R. P., A. Zuckerman, and L. A. Rantz.** 1951. Infection due to chromobacteria; report of 11 cases. AMA Arch Intern Med **88:**461-6.
1140. **White, D.** 2000. The physiology and biochemistry of prokaryotes, 2nd ed. University Press, Oxford, England.
1141. **White, D. G., and P. F. McDermott.** 2001. Biocides, drug resistance and microbial evolution. Curr Opin Microbiol **4:**313-7.
1142. **Whitehead, N. A., A. M. Barnard, H. Slater, N. J. Simpson, and G. P. Salmond.** 2001. Quorum-sensing in Gram-negative bacteria. FEMS Microbiol Rev **25:**365-404.
1143. **Wietzerbin-Falszpan, J., B. C. Das, I. Azuma, A. Adam, J. F. Petit, and E. Lederer.** 1970. Isolation and mass spectrometric identification of the peptide subunits of mycobacterial cell walls. Biochem Biophys Res Commun **40:**57-63.
1144. **Wilfert, J. N., F. F. Barrett, W. H. Ewing, M. Finland, and E. H. Kass.** 1970. *Serratia marcescens*: biochemical, serological, and epidemiological characteristics and antibiotic susceptibility of strains isolated at Boston City Hospital. Appl Microbiol **19:**345-52.
1145. **Wilkowske, C. J., J. A. Washington, 2nd, W. J. Martin, and R. E. Ritts, Jr.** 1970. *Serratia marcescens*. Biochemical characteristics, antibiotic susceptibility patterns, and clinical significance. JAMA **214:**2157-62.
1146. **Williams, D. L., C. Waguespack, K. Eisenach, J. T. Crawford, F. Portaels, M. Salfinger, C. M. Nolan, C. Abe, V. Sticht-Groh, and T. P. Gillis.** 1994. Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob Agents Chemother **38:**2380-6.

1147. **Williams, R. P., and S. M. H. Qadri.** 1980. The pigment of *Serratia*, p. 31–75. *In* A. Von Graevenitz and S. J. Rubin (ed.), The Genus *Serratia*. CRC Press, Inc, Boca Raton, FL.
1148. **Williamson, N. R., H. T. Simonsen, A. K. Harris, F. J. Leeper, and G. P. Salmond.** 2006. Disruption of the copper efflux pump (CopA) of *Serratia marcescens* ATCC 274 pleiotropically affects copper sensitivity and production of the tripyrrole secondary metabolite, prodigiosin. J Ind Microbiol Biotechnol **33:**151-8.
1149. **Willingham, E. M., J. E. Sander, S. G. Thayer, and J. L. Wilson.** 1996. Investigation of bacterial resistance to hatchery disinfectants. Avian Dis **40:**510-15.
1150. **Wilson, D. J., J. H. Kirk, R. D. Walker, and Q. W. Bosworth.** 1990. *Serratia marcescens* mastitis in a dairy herd. J Am Vet Med Assoc **196:**1102-5.
1151. **Wilson, R. W., V. A. Steingrube, E. C. Bottger, B. Springer, B. A. Brown-Elliott, V. Vincent, K. C. Jost, Jr., Y. Zhang, M. J. Garcia, S. H. Chiu, and *et al.*** 2001. *Mycobacterium immunogenum* sp. nov., a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy. Int J Syst Evol Microbiol **51:**1751-64.
1152. **Wilson, T. M., and D. M. Collins.** 1996. *ahpC*, a gene involved in isoniazid resistance of the *Mycobacterium tuberculosis* complex. Mol Microbiol **19:**1025-34.
1153. **Wilson, W. D., S. Mizan, F. A. Tanious, S. Yao, and G. Zon.** 1994. The interaction of intercalators and groove-binding agents with DNA triple-helical structures: the influence of ligand structure, DNA backbone modifications and sequence. J Mol Recognit **7:**89-98.
1154. **Wolinsky, E.** 1992. Mycobacterial diseases other than tuberculosis. Clin Infect Dis **15:**1-10.
1155. **Wolinsky, E.** 1979. Nontuberculous mycobacteria and associated diseases. Am Rev Respir Dis **119:**107-59.
1156. **Wolinsky, E., and T. K. Rynearson.** 1968. *Mycobacteria* in soil and their relation to disease-associated strains. Am Rev Respir Dis **97:**1032-7.
1157. **Wollmann, A., and P. M. Kaulfers.** 1991. Formaldehyde-resistance in *Enterobacteriaceae* and *Pseudomonas aeruginosa*: identification of resistance genes by DNA-hybridization. Zentralbl Hyg Umweltmed **191:**449-56.
1158. **Wolman, S. R.** 1983. Karyotypic progression in human tumors. Cancer Metastasis Rev **2:**257-93.
1159. **Wood, W. I., D. O. Peterson, and K. Bloch.** 1977. *Mycobacterium smegmatis* fatty acid synthetase. A mechanism based on steady state rates and product distributions. J Biol Chem **252:**5745-9.
1160. **Woods, G. L., B. A. Brown-Elliott, E. P. Desmond, G. S. Hall, L. Heifets, G. E. Pfyffer, M. R. Plaunt, J. C. Ridderhof, R. J. Wallace, Jr., N. G. Warren, and F. G. Witebsky.** 2000. Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes; tentative standard 20:M24-T2. p. 218. *In* NCCLS (ed.). Wayne. PA.
1161. **Woodward, H. M., and K. B. Clark.** 1913. A case of infection in man by *Bacterium prodigiosum*. Lancet **1:**314.
1162. **Wright, C. K., S. D. Kominos, and R. B. Yee.** 1976. *Enterobacteriaceae* and *Pseudomonas aeruginosa* Recovered from Vegetable Salads. Appl Environ Microbiol **31:**453–4.
1163. **Wright, E. S., and R. A. Mundy.** 1961. Studies on disinfection of clinical thermometers. II. Oral thermometers from a tuberculosis sanatorium. Appl Microbiol **9:**508-10.
1164. **Wright, J. A., A. K. Chan, B. K. Choy, R. A. Hurta, G. A. McClarty, and A. Y. Tagger.** 1990. Regulation and drug resistance mechanisms of mammalian ribonucleotide reductase, and the significance to DNA synthesis. Biochem Cell Biol **68:**1364-71.
1165. **Wright, N. E., and P. Gilbert.** 1987. Antimicrobial activity of n-alkyltrimethylammonium bromides: influence of specific growth rate and nutrient limitation. J Pharm Pharmacol **39:**685-90.
1166. **Wright, N. E., and P. Gilbert.** 1987. Influence of specific growth rate and nutrient-limitation upon the sensitivity of *Escherichia coli* towards polymyxin B. J Antimicrob Chemother **20:**303-12.
1167. **Wright, P. W., and R. J. J. Wallace.** 1995. Syndromes, diagnosis, and treatment of rapidly growing mycobacteria, p. 373–89. *In* M. D. Rossman and R. R. MacGregor (ed.), Tuberculosis: Clinical management and new challenges. McGraw-Hill Inc, New York.
1168. **Xiong, A., and R. K. Jayaswal.** 1998. Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. J Bacteriol **180:**4024-9.

1169. **Xu, Z., A. L. Horwich, and P. B. Sigler.** 1997. The crystal structure of the asymmetric GroEL-GroES-(ADP) 7 chaperonin complex. Nature **388:**741-50.
1170. **Xu, Z., and P. B. Sigler.** 1998. GroEL/GroES: structure and function of a two-stroke folding machine. J Struct Biol **124:**129-41.
1171. **Yajko, D. M., C. A. Sanders, P. S. Nassos, and W. K. Hadley.** 1990. *In vitro* susceptibility of *Mycobacterium avium* complex to the new fluoroquinolone sparfloxacin (CI-978; AT-4140) and comparison with ciprofloxacin. Antimicrob Agents Chemother **34:**2442-4.
1172. **Yakrus, M. A., S. M. Hernandez, M. M. Floyd, D. Sikes, W. R. Butler, and B. Metchock.** 2001. Comparison of methods for Identification of *Mycobacterium abscessus* and *M. chelonae* isolates. J Clin Microbiol **39:**4103-10.
1173. **Yakrus, M. A., M. W. Reeves, and S. B. Hunter.** 1992. Characterization of isolates of *Mycobacterium avium* serotypes 4 and 8 from patients with AIDS by multilocus enzyme electrophoresis. J Clin Microbiol **30:**1474-8.
1174. **Yamamoto, K., and A. Ishihama.** 2006. Characterization of copper-inducible promoters regulated by CpxA/CpxR in *Escherichia coli*. Biosci Biotechnol Biochem **70:**1688-95.
1175. **Yamamoto, T., A. Ariyoshi, and K. Amako.** 1985. Fimbria-mediated adherence of *Serratia marcescens* strain US5 to human urinary bladder surface. Microbiol Immunol **29:**677-81.
1176. **Yamamoto, T., Y. Tamura, and T. Yokota.** 1988. Antiseptic and antibiotic resistance plasmid in *Staphylococcus aureus* that possesses ability to confer chlorhexidine and acrinol resistance. Antimicrob Agents Chemother **32:**932-5.
1177. **Yamasaki, K., M. Komatsu, T. Yamashita, K. Shimakawa, T. Ura, H. Nishio, K. Satoh, R. Washidu, S. Kinoshita, and M. Aihara.** 2003. Production of CTX-M-3 extended-spectrum beta-lactamase and IMP-1 metallo beta-lactamase by five Gram-negative bacilli: survey of clinical isolates from seven laboratories collected in 1998 and 2000, in the Kinki region of Japan. J Antimicrob Chemother **51:**631-8.
1178. **Yates, M. D., and C. H. Collins.** 1981. Sensitivity of opportunist mycobacteria to rifampicin and ethambutol. Tubercle **62:**117-21.
1179. **Yates, M. D., A. Pozniak, A. H. Uttley, R. Clarke, and J. M. Grange.** 1997. Isolation of environmental mycobacteria from clinical specimens in south-east England: 1973-1993. Int J Tuberc Lung Dis **1:**75-80.
1180. **Yatsuyanagi, J., S. Saito, T. Konno, S. Harata, N. Suzuki, J. Kato, and K. Amano.** 2006. Nosocomial outbreak of ceftazidime-resistant *Serratia marcescens* strains that produce a chromosomal AmpC variant with N235K substitution. Jpn J Infect Dis **59:**153-9.
1181. **Yerushalmi, H., M. Lebendiker, and S. Schuldiner.** 1995. EmrE, an *Escherichia coli* 12-kDa multidrug transporter, exchanges toxic cations and H+ and is soluble in organic solvents. J Biol Chem **270:**6856–63.
1182. **Yethon, J. A., E. Vinogradov, M. B. Perry, and C. Whitfield.** 2000. Mutation of the lipopolysaccharide core glycosyltransferase encoded by *waaG* destabilizes the outer membrane of *Escherichia coli* by interfering with core phosphorylation. J Bacteriol **182:**5620-3.
1183. **Yi, W., L. Zhu, H. Guo, M. Li, J. Li, and P. G. Wang.** 2006. Formation of a new O-polysaccharide in *Escherichia coli* O86 via disruption of a glycosyltransferase gene involved in O-unit assembly. Carbohydr Res **341:**2254-60.
1184. **Young, I. G., and F. Gibson.** 1969. Regulation of the enzymes involved in the biosynthesis of 2,3-dihydroxybenzoic acid in *Aerobacter aerogenes* and *Escherichia coli*. Biochim Biophys Acta **177:**401-11.
1185. **Yu, V. L.** 1979. *Serratia marcescens*: historical perspective and clinical review. N Engl J Med **300:**887-93.
1186. **Yuan, Y., and C. E. Barry, 3rd.** 1996. A common mechanism for the biosynthesis of methoxy and cyclopropyl mycolic acids in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA **93:**12828-33.
1187. **Yuan, Y., D. C. Crane, J. M. Musser, S. Sreevatsan, and C. E. Barry, 3rd.** 1997. MMAS-1, the branch point between *cis*- and *trans*-cyclopropane-containing oxygenated mycolates in *Mycobacterium tuberculosis*. J Biol Chem **272:**10041-9.
1188. **Yuan, Y., R. E. Lee, G. S. Besra, J. T. Belisle, and C. E. Barry, 3rd.** 1995. Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA **92:**6630-4.

1189. **Zeelie, J. J., and T. J. McCarthy.** 1998. Effects of copper and zinc ions on the germicidal properties of two popular pharmaceutical antiseptic agents cetylpyridinium chloride and povidone-iodine. Analyst **123:**503-7.
1190. **Zeiler, H. J.** 1985. Evaluation of the in vitro bactericidal action of ciprofloxacin on cells of *Escherichia coli* in the logarithmic and stationary phases of growth. Antimicrob Agents Chemother **28:**524-7.
1191. **Zeiler, H. J., and R. Endermann.** 1986. Effect of ciprofloxacin on stationary bacteria studied *in vivo* in a murine granuloma pouch model infected with *Escherichia coli*. Chemotherapy **32:**468-72.
1192. **Zenone, T., A. Boibieux, S. Tigaud, J. F. Fredenucci, V. Vincent, and D. Peyramond.** 1998. Nontuberculous mycobacterial tenosynovitis: report of two cases. Clin Infect Dis **26:**1467-8.
1193. **Zgurskaya, H. I., and H. Nikaido.** 2000. Multidrug resistance mechanisms: drug efflux across two membranes. Mol Microbiol **37:**219-25.
1194. **Zhang, L., P. J. Murphy, A. Kerr, and M. E. Tate.** 1993. *Agrobacterium conjugation* and gene regulation by N-acyl-L-homoserine lactones. Nature **362:**446-8.
1195. **Zhang, Y., T. Garbe, and D. Young.** 1993. Transformation with *katG* restores isoniazid-sensitivity in *Mycobacterium tuberculosis* isolates resistant to a range of drug concentrations. Mol Microbiol **8:**521-4.
1196. **Zhao, W. H., Z. Q. Hu, G. Chen, K. Matsushita, K. Fukuchi, and T. Shimamura.** 2007. Characterization of imipenem-resistant *Serratia marcescens* producing IMP-type and TEM-type beta-lactamases encoded on a single plasmid. Microbiol Res **162:**46-52.