The Molecular Basis for Preservative Resistance in *Burkholderia cepacia* Complex Bacteria

Thesis presented for the Degree of Philosophiae Doctor
By

Laura Thomas, BSc (Hons) Microbiology

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Organisms and the Environment Division
Cardiff School of Biosciences

Cardiff University

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The plural of anecdote is not data.

Frank Kotsonist, Pharmacologist

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SCIENTIFIC CONFERENCES AND AWARDS

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SUMMARY

Burkholderia cepacia complex bacteria can contaminate and survive in a variety of antimicrobial and preserved industrial products. Contamination may lead to economic loss for manufacturers and also potentially pose a risk to the health of vulnerable consumers. Understanding the interaction between Bcc bacteria and preservatives, and the molecular basis for their resistance, is essential in order to better target these organisms and to facilitate the implementation of improved preservative strategies which target resistance mechanisms.

In the present study, multi-locus sequence typing analysis of a collection of 67 Bcc isolates from environmental-industrial sources was used to expand the current knowledge of Bcc species diversity within this niche and identified *B. lata* (n=17) and *B. cenocepacia* (n=11) as predominant species groups. The relationship between Bcc species diversity, isolation source and preservative susceptibility was investigated using a collection of 83 genetically diverse Bcc strains from clinical, environmental and environmental-industrial isolation sources. Susceptibility to eight preservatives was not related to Bcc taxonomy, as susceptibility profiles varied both between and within species groups. However, Bcc isolates from environmental-industrial sources had a significantly higher minimum inhibitory and minimum bactericidal concentration (MIC and MBC) for the formaldehyde releasing agent DMDM hydantoin. This suggests that for this preservative agent, susceptibility was related to source and that the selection of highly tolerant Bcc bacteria had occurred within the niche. Isothiazolone, DMDM hydantoin, phenoxyethanol and methyl paraben preservatives were observed to be highly efficacious against Bcc bacteria when evaluated in growth medium at the maximum concentration regulated for use in rinse-off personal care products in EU-regulated countries. Benzethonium chloride and sodium benzoate preservatives had the weakest anti-Bcc activity at these levels but were effective against several strains. Combinations of preservatives, and preservatives with potentiating agents, were evaluated for synergistic anti-Bcc activity. The greatest anti-Bcc activity was observed when isothiazolone preservatives were combined with EDTA or phenoxyethanol, with each combination resulting in an additive effect. The competency of Bcc bacteria to adapt to preservatives was explored via the progressive sub-culture of *B. lata* strain 383 in sub-inhibitory preservative concentrations. This genome sequence strain represented a Bcc species commonly encountered in the environmental-industrial niche. Stable adaptive-resistance to isothiazolone and benzethonium chloride preservatives was developed. Phenoxyethanol, DMDM hydantoin and methyl paraben preservatives were recalcitrant to *B. lata* strain 383 adaptation. The preservative and antibiotic susceptibility profiles of the adapted *B. lata* strain 383 derivatives differed, suggesting the induction of agent-specific adaptive-resistance mechanisms had occurred. The *B. lata* 383-CMIT,-BIT, derivatives (adapted respectively to chloromethylisothiazolinone and benzisothiazolinone), demonstrated cross-resistance to isothiazolone preservatives and fluoroquinolone antibiotics. Sequence analysis of the topoisomerase genes in these derivatives revealed fluoroquinolone resistance was not mediated by target modification. Preservative-induced adaptive resistance was not associated with overall increased multi-drug resistance.

The molecular basis for resistance to DMDM hydantoin and isothiazolone preservatives was investigated via the random transposon mutagenesis of *B. lata* strain 383 using pTn*Mod*OTp'. Several genetic pathways were identified as putative preservative resistance determinants, suggesting that resistance is multi-factorial. These included the detoxification of formaldehyde via a glutathione-dependent pathway; a type II general secretory system (A3244_A3233 genes); a homologue of an ABC-type efflux system involved in resistance to organic solvents (A3512_A3517 genes); homologues of multi-drug RND-type efflux systems EmrB/QacA-Emr-TolC; and bacterial defence mechanisms against oxidative stress.

A transcriptomic microarray-based approach was used to profile global gene expression of *B. lata* strain 383 in response to sub-MIC of 0.00162% DMDM hydantoin and 0.00001498% of a methylisothiazolinone and CMIT blend, as well as isothiazolone-induced adaptive resistance. With a 1.5-fold change and $P < 0.05$ confidence level criterion applied, few significant changes were observed after a single sub-MIC exposure, and the differential expression of putative resistance determinants identified by transposon mutagenesis was not induced at these concentrations. Isothiazolone-induced adaptive-resistance involved a greater number of significant gene expression changes that were stable irrespective of the presence of the priming agent, with 126 up-regulated and 90 down-regulated genes. Transcriptomic analysis suggested that isothiazolone-induced adaptive resistance was multi-factorial in nature, and identified active efflux as a putative key resistance mechanism. A novel role for a RND-type efflux system (B1004_B1006 genes) was identified, and the up-regulation of the ABC-efflux system (A3512_A3517 genes) and bacterial defence mechanisms against oxidative stress corroborated the transposon mutagenesis findings. *B. lata* strain 383-CMIT demonstrated a four-fold reduction in MIC for the priming preservative (2.81E-04%) in the presence of 512 mg/L of the efflux inhibitor PAβN. Resistance mechanism targeted preservative strategies such as using efflux inhibitors may work to improve preservation.

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
AHL	Acyl-homoserine-lactone
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
a_w	Water activity
BC	Benzethonium chloride
Bcc	*Burkholderia cepacia* complex
BIT	Benzisothiazolinone
BLAST	Basic local alignment tool
Bp	Base pairs
BSM	Basal salts media
cDNA	Complementory DNA
CDS	Coding sequences
CF	Cystic Fibrosis
CFTA	Cosmetics, Toiletry and Fragrance society
CFU / ml	Colony forming units per millilitre
CFU/g	Colony forming units per gram
CG	Capryl glycol
cGMP	Current good manufacturing procedures
ci-di-GMP	Cyclic diguanosine monophosphate
CIP	Ciprofloxacin
CLIN	Clinical
CLSI	The Clinical and Laboratory Standards Institute
CMIT	Chloromethylisothiazolinone
Colipa	European Cosmetics, Tolietries & Perfumery Association
C_T	Cycle threshold
CYG	Casamino acids, yeast extract, glucose
DMDM	Dimethylol dimethyl
DMH	Dimethylol dimethyl hydantoin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EEC	European Economic Community
EG	Ethylhexyl glycerin
ENV	Environmental
ENVI	Environmental Industrial

EP	European Pharmacopeia
EPI	Efflux pump inhibitor
EPS	Exopolysaccharide
EU	European Union
FA	Formaldehyde
FDA	Foods and Drugs Administration
FIC	Fractional inhibitory concentration
fw	Forward primer
GSH-FDH	Gluthione-dependent formaldehyde dehydrogenase
HCL	Hydrochloric acid
IG	Intergenic
INCI	International Nomenclature of Cosmetic Ingredients
ISO	Isosensitest
Kdo	3-deoxy-D-manno-octo-2-ulsonic acid
Km	Kanamycin
LB	Luria-Bertani
LiCl	Lithium chloride
LMG	Belgian co-ordinated collections of micro-organisms, Ghent.
LPS	Lipopolysaccharide
LVX	Levofloxacin
MATE	Multidrug and toxic-compound extrusion
MBC	Minimum bactericidal concentration
MDR	multidrug resistance
MFS	Major facillitator superfamiliy
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MIT	Methylisothiazolinone
MIT/CMIT	3:1 mixture of chloro to non-chlorinated isothiazolone forms
MLST	Multi-locus sequence typing
MP	Methyl paraben
MPR	Multi-preservative resistance
N_2	Nitrogen
NaOH	Sodium hydroxide
NCBI	National Centre of Biotechnology and Information
NCTC	National Collection of Type Cultures
NOR	Norfloxacin
NTA	Nitrilotriacetic acid

OD	Optical density
ORNL	Oak Ridge National Laboratory
PAβN	L-Phe-Arg-β-napthylamide
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PH	Phenoxyethanol
PMF	Proton motive force
Pmx	Polymyxin B
PP	Propyl paraben
QAC	Quarternary ammonium compounds
QRDR	Quinolone resistance determining region
qRT-PCR	Quantitative real-time PCR
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA
RAPEX	Rapid Alerts System for non-food consumer products in EU countries
RFLP	Restricted fragment length polymorphism
RNA	ribonucleic acid
RND	Resistance nodulating division
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcriptase PCR
rv	Reverse primer
SB	Sodium benzoate
SD	Standard Deviation
SMR	small multidrug resistance
Spp.	Species
SPX	Sparfloxacin
ST	Sequence type
Taq	*Thermus aquaticus*
Tc	Tetracycline
TCS	Two-component regulatory systems
Tn	Transposon
Tp	Trimethoprim
TSA	Tryptone soya agar
TSB	Tryptone soya broth
UV	Ultra violet
WT	Wild-Type

1 INTRODUCTION

1.1 MICROBIAL CONTAMINATION IN THE PERSONAL CARE INDUSTRY

The microbiological safety of commercial goods is of great importance to industries as microbial spoilage can lead to economic loss and may, depending on the contaminating organism, pose a risk to consumer health. The personal care industry, producers of cosmetics and toiletries, manufactures products used daily by consumers on a global scale (www.colipa.com). Personal care products are used to cleanse, perfume, beautify or decorate the human body and are mainly applied to the skin, mouth or hair (Perry, 2001). Although these products are not intended to permanently alter physiological attributes, some products contain active ingredients with medicinal claims e.g. anti-dandruff shampoos and anti-aging creams (Orth *et al.*, 2006).

In contrast to the pharmaceutical industry, where sterile products are often essential, personal care products are not manufactured or marketed as sterile. However, the products must not contain unacceptable levels or types of microorganisms at the time of purchase, or during use (Farrington *et al.*, 1994). There are no formal regulations defining the number of microorganisms that are acceptable in cosmetic products (Orth *et al.*, 2006), but it is widely accepted that the microbial bioburden of a product should not cause damage to human health or cause its properties to change. Many manufacturers follow internal guidelines based on recommendations developed by professional associations that define general specifications based on product type and identify harmful microorganisms that must not be present (Orth *et al.*, 2006) . Guidelines by The European Cosmetics Toiletries & Perfumery Association (Colipa) recommend that baby and eye products have less than 10^2 CFU/g, and all other products less than 10^3 CFU/g. For both product types, the guidelines recommend the absence of *P. aeruginosa, S. aureus* and *C. albicans* (Orth *et al.*, 2006). Considering that viable organisms have the potential to grow in the finished product, it is most appropriate that the microbial limit be as low as possible.

Manufacturers strive to achieve high standards of production and many follow internal guidelines of current Good Manufacturing Practices (cGMP), based on recommendations published by professional associations. Guidelines describe methods and practices relating to all aspects of production including plant hygiene and sanitation, maintenance of plant machinery, and environmental monitoring. GMPs also relate to the assessment, quality control, handling and storage of raw materials. Generally, very few sterile raw materials

are used in the production of non-sterile personal care products; and consequently, each material contains an inherent microbial burden that contributes to the overall bioburden of a product (Orth *et al.*, 2006). cGMPs should ensure that products, whilst not necessarily sterile, do not become contaminated with unacceptable levels or types of microorganisms (Perry, 2001). However, even strict compliance to cGMPs cannot entirely prevent microbial contamination from occurring during manufacture. Preservative agents are incorporated into raw materials and finished product to inhibit proliferation of spoilage organisms that may gain access to the process stream or through consumer use (the preservation of personal care products is discussed in section 1.3).

The growth and proliferation of contaminating microorganisms often depends on the raw materials and the physicochemical properties of the formulation for example, water activity (a_w; the amount of freely available water), pH and nutrients (Orth *et al.*, 2006). Most bacteria are only able to proliferate at water activity levels above 0.9, and in formulations with a pH between 5.5 and 8.5; manipulation of either or both parameters can suppress microbial growth (Orth *et al.*, 2006). However, some bacteria are tolerant of low water activity for example *Staphylococcus aureus* (a_w0.86); while others, for example *Burkholderia cepacia* complex (Bcc) bacteria, have been recovered from formulation with a low pH of $\leq$ 3.2 (Borovian, 1983). Many personal care products have a high water activity and abundance of microbial nutrients, making them particularly susceptible to microbial growth (Orth *et al.*, 2006; Orus & Leranoz, 2005).

1.1.1. The consequences of microbial contamination

Depending on the contaminating microorganisms, active proliferation can lead to undesirable changes to the physiochemical and/or organoletpic properties. For example, microbial growth can alter the colour of a product (via production of pigment or changes in the pH), or alter its viscosity. Gross contamination of product by gas-forming organisms may decrease the internal pressure of a container, causing it to swell and even explode; while facultative aerobes may cause an internal decrease in pressure causing the side panels of packaging to bow (Orth *et al.*, 2006).

The cost to the manufacturers depends on the extent of contamination and the speed at which it is detected. At best, early detection may result in the loss of contaminated raw materials, a batch of product, and the associated expense of sanitising affected plant equipment and production lines. Delayed detection can result in the contamination of

additional batches of the same or other product that may have been produced on a single contaminated production line, and potentially, a recall of contaminated products from the supply chain. At worst, a public recall of contaminated goods may be required. For example in 2009, the BBC news reported a public recall of 120,000 bottles of Vicks Sinex nasal spray manufactured by Proctor & Gamble, contaminated with low levels of the opportunistic pathogen *Burkholderia cepacia.* In addition to the aforementioned financial loss associated with an instance of contamination, the negative publicity of a public recall may additionally harm the reputation of the company and/or brand name.

Generally the use of contaminated personal care products does not cause infection in healthy individuals (Orth *et al.*, 2006). However, there have been reports in which contaminated product has been determined as the source of infection in vulnerable individuals. The type of organism, microbial load (infective dose), site of application, host immune status and/or degree of tissue damage at the site of application are all major factors that determine whether use of a contaminated product may result in infection (Russell, 2004b). Naturally, there is an increased risk of injury to the consumer associated with contaminating organisms considered to be opportunistic pathogens e.g. *S. aureus*, *P. aeruginosa, Candida albicans* and *B. cepacia* complex bacteria. Non-pathogenic microorganisms are not generally considered to be a health risk; however, contaminated product may cause injury if applied to broken skin or the eyes (Orth *et al.*, 2006). In most cases where use of contaminated product has been associated with injury to the consumer, the microbial load has exceeded 10^5 CFU/g (Orth *et al.*, 2006). Whilst the microbial limit is not strictly regulated it is fair to presume that manufacturers would not knowingly release product with such a high microbial burden. Such burdens are likely to be a result of errors in quality control testing, allowing the release of contaminated product, with levels of contaminating microorganisms below the detection limit at the time of testing, that increased subsequently: a situation commonly referred to as the 'phoenix phenomenon' (Orth *et al.*, 2006).

1.1.2. Key contaminants in the personal care industry

Methods of detecting microbial contamination in raw materials and finished product usually rely on classical microbiological approaches such as the traditional plate count (Orus & Leranoz, 2005). The recovery of contaminants can be challenging. Viable cells that have sustained injury by manufacturing conditions and/or preservative agents may enter a viable but non-culturable state, preventing proliferation on nutrient-rich agar

(Oliver, 2005; Orus & Leranoz, 2005). Newer methods such as bioluminescence, impedance testing and cytometry are based on the metabolic state of a microorganism and offer both rapid and reliable detection of stressed cells (Orth *et al.*, 2006). However, at present, rapid methods cannot detect specific groups of microorganisms. The species identification of cultured isolates is commonly based on phenotypic and biochemical tests.

The microbiology of personal care products is complex, due to a wide range of product formulations, manufacturing procedures and conditions of consumer use (Perry, 2001). A diverse range of yeasts, moulds and bacteria may be encountered; and often, it is the physiochemical properties of a product that dictates the type of contaminating organism(s). Organisms frequently isolated from inadequately preserved aqueous personal care products include *Klebsiella, Enterobacter, Staphylococcus* and *Bacillus* species, *Pseudomonas,* Bcc species, *Penicillium* and *Candida albicans* (Perry, 2001). Gram-negative bacteria are most commonly encountered, often introduced to the process stream through water supplies (Jimenez, 2007; Perry, 2001).

Literature on microbial contamination of personal care products is somewhat limited as the publication of instances of contamination is not a priority for manufacturers. However, product recalls reported by the Food and Drug Administration (FDA) and published notifications by the Rapid Alerts System for non-food consumer products in EU countries (RAPEX) reveal *P. aeruginosa* and *B. cepacia* to be the predominant Gram negative contaminants in the personal care industry (Jimenez, 2007; Lundov & Zachariae, 2008; Wong *et al.*, 2000). Jimenez (2007) described the microbial diversity of 134 non-sterile and 193 sterile pharmaceutical (including personal care) product recalls reported by the FDA between 1998 to 2006. Of these non-sterile recalls, 60% were associated with contamination by Gram-negative bacteria; and only 4% were associated with Gram-positives. Further analysis revealed that either *Pseudomonas, B. cepacia* or *Ralstonia pickettii* contamination, had accounted for 48% of the non-sterile recalls; yeast or mould contamination were found in 23% of products. Sterile product recalls were mainly due to lack of sterility assurance; however, 7% were associated with yeast and 6% with Gram-negative contamination. *"B. cepacia"* was the most frequently reported microbial species, accounting for 22% and 2.5% of sterile and non-sterile product recalls respectively. This also reflected an additional weakness of identification and reporting in the manufacturing sector, few reports accurately document the exact species within the *B. cepacia* complex group of organisms (Mahenthiralingam *et al.*, 2008).

1.2 THE BURKHOLDERIA CEPACIA COMPLEX

1.2.1 Taxonomy and current diversity

In 1950, Walter Burkholder published a description of *Pseudomonas cepacia* as the causative agent "sour skin" or "slippery skin", a disease of harvested onion bulbs (Burkholder, 1950). In subsequent years, two additional pseudomonads were found to be synonymous to *P. cepacia:* an environmental isolate from soil and water in Trinidad, later classified as *P. multivorans* (Stanier *et al.*, 1966), and a group of opportunistic pathogens known as "Eugenic oxidisers group I" but later classified as *P. kingii* (Jonsson, 1970). With the advent of rRNA-DNA hybridisation and RNA sequencing methodology, the taxonomy of established genera, including *Pseudomonas* began to change (Vandamme & Dawyndt, 2011). In 1973, Palleroni *et al.* revealed that the genus *Pseudomonas* consisted of five major species clusters based on ribosomal RNA homologies (*P. cepacia* was assigned to rRNA group II) (Palleroni *et al.*, 1973) and that these clusters formed a phylogenetic part of a major group of (Gram-negative) bacteria now known as the *Proteobacteria* (Vandamme & Dawyndt, 2011). In 1992, Yabuuchi *et al.* (1992) proposed the transfer and reclassification of *P. cepacia* and six other species within the *Pseudomonas* rRNA group II, to a new genus *Burkholderia*, with *Burkholderia cepacia* as the type species. The revisions proposed by Yabuuchi *et al.* (1992) were based on a limited number of strains within the *Pseudomonas* rRNA group II. As a result, additional species were reclassified as *Burkholderi*a in subsequent studies, and two classified as *Burkholderia* were reclassified into the novel genus *Ralstonia* (Vandamme & Dawyndt, 2011). To date the *Burkholderia* genus has 60 formally named species, several candidate species and looks to continue expanding (Vandamme & Dawyndt, 2011).

During the 1990s, researchers noticed an interesting heterogeneity among strains identified as *Burkholderia cepacia* using traditional and molecular typing approaches (Coenye, 2007b). A collaborative polyphasic-taxonomic approach (i.e. biochemical, molecular and genetic tests) revealed that *B. cepacia* isolates from Cystic fibrosis (CF) patients , other human clinical samples and the environment were closely related but genetically distinct; and that isolates belonged to at least five genomic species or genomovars (i.e. a phenotypically similar genomic species (Ursing *et al.*, 1995; Vandamme *et al.*, 1997). Subsequent studies have discovered additional genomovars and novel species belonging to this group, collectively referred to as the *Burkholderia cepacia* complex (Bcc). With the evolution of phenotypic tests capable of distinguishing between the genomic species,

genomovar were replaced with formal species names. To date, there are 17 validly named species within the complex (Table 1, Figure 1); however, published and unpublished data from recent typing studies demonstrate there is still a considerable number of unnamed species to be resolved (Vandamme & Dawyndt, 2011).

Bcc strains share a high degree of 16S rRNA and *recA* (recombinase A) gene sequence similarity, of 98-100% and 94-95% respectively. As a group, Bcc species share moderate levels of DNA-DNA hybridisation (30-50%) whereas strains of the same species usually have ≥70% relatedness (Coenye, 2007b). Differentiation of the closely related and phenotypically similar species within the complex can be particularly problematic, often requiring the use of molecular techniques for definitive identification (Coenye *et al.*, 2001c).

1.2.2 Identification of Bcc species

Burkholderia are aerobic, non-spore forming straight or slightly curved Gram-negative rods measuring 1 to 5 µm in length and 0.5 to 1.0 µm in width (Holt, 1994). Most species are motile and have one or more polar flagella. They are catalase positive and most weakly to strongly oxidase positive. All appear as non-fermenters on MacConkey agar, most degrade glucose oxidatively and degrade nitrite to nitrate or nitrogen gas. *Burkholderia* have exceptional nutritional versatility, able to utilise a variety of carbon sources. As mesophiles, the optimum growth temperature for most strains is between 30 and 37°C (Coenye & Vandamme, 2007b).

Differentiation of the closely related species of the complex using conventional phenotypic or biochemical test alone, is particularly problematic; many commercial bacterial identification kits (e.g. API 20NE and Vitek) cannot reliably distinguish between them (Henry *et al.*, 2001; Shelly *et al.*, 2000). All *Burkholderia* species have a degree of phenotypic variation, often influenced by substrate and growth conditions. Phenotypic variation has also been observed in sequential isolates of the same strain (Mahenthiralingam *et al.*, 2008). Only *B. multivorans* and *B. stabilis* can be distinguished from other species by relatively simple biochemical tests (Henry *et al.*, 2001). Coenye *et al.* (2001c) recommended the use of selective agars, based on their nutritional versatility and intrinsic resistance to various antimicrobial agents, in conjunction with biochemical tests to improve the accuracy of identification and, more importantly, to avoid lack of identification, essential in a clinical context.

Historic difficulties associated with identifying Bcc species have driven the development of molecular and genetic tools capable of reliably identifying and differentiating between species (Coenye & Vandamme, 2007b). Analysis of full length 16S rRNA gene sequences can distinguish all formally named Bcc species (Mahenthiralingam *et al.*, 2008). However, partial 16S rRNA gene sequencing and restriction fragment length polymorphism (RFLP) analysis with multiple enzymes are unable to distinguish reliably all Bcc species due to insufficient sequence variation (Coenye & Vandamme, 2007b). Techniques exploiting species specific sequence polymorphisms in the *recA* gene have proved far more discriminatory. *RecA*-based PCR assays can identity all *Burkholderia* (i.e. at the genus level) (Mahenthiralingam *et al.*, 2000a; Vermis *et al.*, 2002) or species level within the complex (i.e. as a group) (Payne *et al.*, 2005) . However, considering the discovery of new Bcc diversity and taxonomic refinement of the complex since the design of the original primers, it is questionable whether such PCR should be considered absolutely specific (Mahenthiralingam *et al.*, 2008).

Analysis of a single locus gene such as *recA* can provide excellent discrimination, but is limited to the species level (Baldwin *et al.*, 2005). Techniques capable of discriminating beyond the species level include pulse-field gel electrophoresis (PFGE) of macrorestriction fragments of chromosomal DNA (Coenye & Vandamme,, 2007b), random amplified polymorphic DNA (RAPD) fingerprinting (Mahenthiralingam *et al.*, 1996) and BOX elements (BOX-PCR) fingerprinting (Coenye *et al.*, 2002). Although highly discriminatory, these methods are relatively time-consuming, require a degree of skill and also depend on a subjective comparison of banding profiles that impact on interlaboratory comparison of data. Multilocus sequence typing (MLST) is a relatively new technique that surpasses single gene sequence-based methods and offers superior discrimination in a single approach without using polyphasic techniques. In 2005, Baldwin *et al.* developed an MLST scheme for the Bcc. In brief, MLST examines nucleotide polymorphorisms in seven housekeeping genes that possess a low rate of mutation and recombination, that are located on the first and second chromosomes: (*altp*, ATP synthase β-chain; *gltB*, glutamate synthase large subunit; *gyrB*, DNA gyrase ; *recA*, recombinase A; *lepA*, GTP-binding protein; *phaC*, acetoacetyl-CoA reductase and *trpB*, tryptophan synthase)(Baldwin *et al.*, 2005). Sequence data is searched against a public MLST database (http://pubmlst.org/bcc/) and nucleotide differences are assigned an allele number, the resulting allelic profile being given a sequence type (ST) number that is unique to each strain. This method enables

interlaboratory comparison of data and has proved to be a valuable epidemiological surveillance tool (Urwin & Maiden, 2003).

Table 1. Current diversity of the *Burkholderia cepacia* complex

Species	Other designation	Reference
Burkholderia cepacia	*B. cepacia* Genomovar I	(Vandamme *et al.*, 1997)
Burkholderia multivorans	*B. cepacia* Genomovar II	(Vandamme *et al.*, 1997)
Burkholderia cenocepacia	*B. cepacia* Genomovar III	(Vandamme *et al.* 1997, 2003*)*
Burkholderia stabilis	*B. cepacia* Genomovar IV	(Vandamme *et al.*, 1997 ; 2000)
Burkholderia vietnamiensis	*B. cepacia* Genomovar V	(Gillis *et al.*, 1995 ; Vandamme *et al.*, 1997)
Burkholderia dolosa	*B. cepacia* Genomovar VI	(Coenye *et al.*, 2001a; Vermis *et al.*, 2004)
Burkholderia ambifaria	*B. cepacia* Genomovar VII	(Coenye *et al.*, 2001b)
Burkholderia anthina	*B. cepacia* Genomovar VIII	(Vandamme *et al.*, 2002)
Burkholderia pyrrocinia	*B. cepacia* Genomovar IX	(Vandamme *et al.*, 2002)
Burkholderia ubonensis	*B. cepacia* Genomovar X	(Vanlaere *et al.*, 2008; Yabuuchi *et al.*, 2000)
Burkholderia latens		(Vanlaere *et al.*, 2008)
Burkholderia diffusa		(Vanlaere *et al.*, 2008)
Burkholderia arboris		(Vanlaere *et al.*, 2008)
Burkholderia seminalis		(Vanlaere *et al.*, 2008)
Burkholderia metallica		(Vanlaere *et al.*, 2008)
Burkholderia contaminans		(Vanlaere *et al.*, 2009)
Burkholderia lata		(Vanlaere *et al.*, 2009)

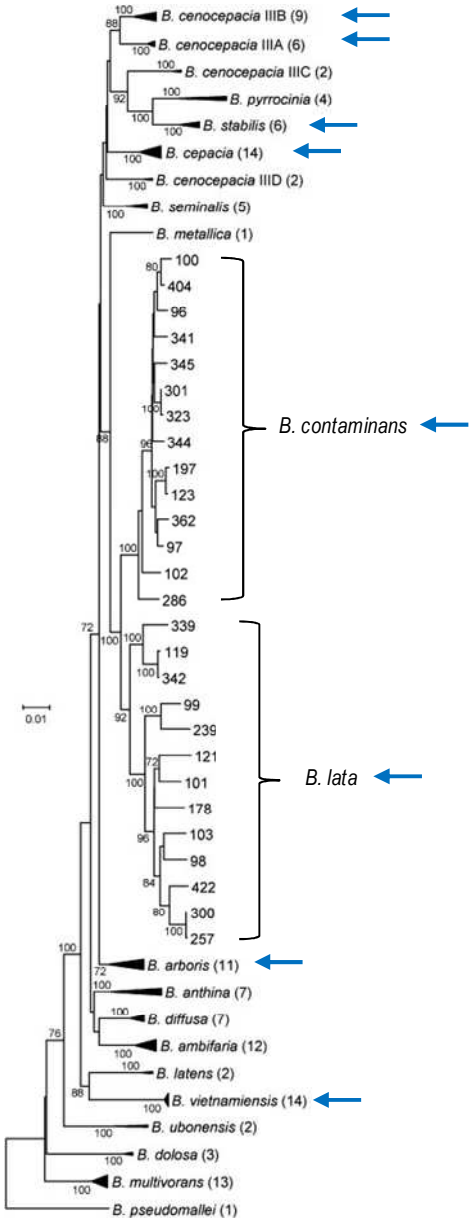


Figure 1. The diversity and relationship between species of the *B. cepacia complex* as revealed by MLST phylogenetic analysis.

A phylogenetic tree of concatenated nucleotide sequences from seven MLST target loci (*atpD*, *gltD*, *gyrB*, *recA*, *lepA*, *phaC*, and *trpB*) of Bcc bacteria, using the neighbour-joining method. Established species names are shown; different *B. cenocepacia recA* lineages are designated IIIA, IIIB, IIIC and IIID (Vandamme *et al.* 2003); and the numbers of *B. contaminans* and *B. lata* sequence types included in the analysis are included to show diversity within these groups. Bootstrap values greater than 70% are shown for 100 replicates, with *B. pseudomallei* as an outgroup. Blue arrows indicate species groups commonly encountered as environmental-industrial contaminants. Adapted from Vanlaere *et al.*, 2009.

1.2.3 Bcc in the natural environment

Bcc bacteria are widespread in the natural environment, occupying a diverse number of ecological niches, including soil, fresh water bodies and the rhizosphere of crop plants (Vial *et al.*, 2011). Considered one of the most versatile groups of Gram-negative bacteria, the Bcc owe much of their versatility in disease and natural biology to their vast genetic capacity (Coenye & Vandamme, 2007b). Bcc species have a characteristic large genome averaging 7.5 Mb in size, that is typically organised into two to four large replicons (defined as chromosomes), and may harbour additional plasmids. Another feature of the Bcc genome is a high plasticity, as insertion sequences (acquired via horizontal transfer) promote rearrangements between replicons (Coenye & Vandamme, 2007b). The characterisation of Bcc species diversity and distribution in the natural environment has been greatly overshadowed by investigation of their clinical epidemiology (discussed in section 1.2.4). Further to this, Bcc distribution and prevalence within ecological niches has not been equally investigated, attention has been largely focused on the rhizosphere of commercially important crops (Vial *et al.*, 2011).

1.2.3.1 Aquatic environments

The characterisation of Bcc in aquatic environments is relatively limited (Vial *et al.*, 2011). Members of the Bcc have been isolated from fresh water systems and sediments, and it is thought natural waters may have a high species diversity. For example, *B. cepacia*, *B. cenocepacia*, and *B. vietnamiensis* were among five genomovars recovered from two European rivers (Vermis *et al.*, 2003; Vial *et al.*, 2011). Salt water bodies are not generally considered a natural reservoir of Bcc bacteria, despite the groundbreaking discovery of the *Burkholderia* SAR-1 genome in the metagenomic study of the Sargasso Sea (Venter *et al.*, 2004). Subsequent studies have since revealed that Bcc isolates, including an identical cultivable clone of SAR-1 (*B. contaminans* SAR-1), grow poorly in sea water and culture media with equivalent salt concentrations, suggesting the ocean is not their preferred habitat. The presence of the *Burkholderia* SAR-1 sequence in the Sargasso Sea is considered by some to be a result of a contamination in the original metagenomic survey (Mahenthiralingam *et al.*, 2006).

1.2.3.2 Phytopathogenic Bcc

As previously discussed, *Burkholderia* were first described as a phytopathogen responsible for “sour skin “or “slippery skin” rotting diseases of harvested onions, in which infected onion bulbs become yellow/brown in colour, are sour smelling, and display extensive

tissue maceration (mediated by a plasmid-encoded pectate hydrolase enzyme system) (Mahenthiralingam *et al.*, 2005). *B. cepacia* has historically been associated with the onion rot disease. However, subsequent studies have since shown that onion bulbs inoculated with strains of *B. cenocepacia, B. vietnamiensis* and *B. pyrrocinia* isolated from onion rhizosphere, demonstrate the characteristic tissue maceration (Jacobs *et al.*, 2008). Species of the Bcc are not generally considered to be important plant pathogens, in comparison to other *Burkholderia* species such as *B. glumae* and *B. plantari*, which rot rice grains and cause other commercially important crops to wilt (Jeong *et al.*, 2003; Mahenthiralingam *et al.*, 2005). However, *B. cenocepacia* and uncharacterised Bcc genovar(s) have been described as the causative agents of fingertip rot in bananas, and soft rot in apricots (Fang *et al.*, 2009; Lee & Chan, 2007).

1.2.3.3 Soil and the plant rhizosphere

In contrast to their occasional pathogenicity, most Bcc species in the natural environment are considered highly beneficial. Although regarded as dominant components of soil ecosystems members of the Bcc are more commonly associated with the plant root system, often reported as dominant bacteria in the rhizosphere of important commercial crops (e.g. rice, maize, pea and cotton) (Vial *et al.*, 2011). The characterisation of Bcc distribution in the rhizosphere is still somewhat limited to maize plants. Commonly encountered Bcc species include *B. cepacia, B. cenocepacia, B. ambifaria, B. stabilis, B. pyrrocinia* and *B. dolosa* (Dalmastri *et al.*, 2007; Payne *et al.*, 2005; Ramette *et al.*, 2005). However, studies have revealed geographical differences in the distribution of Bcc species between maize rhizospheres sampled at different locations. For example, *B. ambifaria*, the most commonly encountered species group associated with maize cultivated in the USA and Italy (Dalmastri *et al.*, 2007; Ramette *et al.*, 2005), was not detected in the rhizosphere of maize cultivated in China, where *B. cenocepacia* was the dominant species recovered (Zhang & Xie, 2007).

The presence of Bcc isolates in the rhizosphere, and in some cases within plant tissue, of some commercial crops has been demonstrated to promote growth and yield (Vial *et al.*, 2011). Bcc bacteria can exert a direct beneficial effect on plant growth by fixing atmospheric nitrogen (N_2), synthesising phytohormones, altering endogenous plant ethylene levels and/or by solubilising inorganic phosphates. Bcc can also exert an indirect benefit on plant growth by the production of antibiotic and/or antifungal compounds, that protect the crop against soilborne plant pathogens (Compant *et al.*, 2008). The potential

benefits of harnessing such protective properties led to the registration of certain Bcc strains for commercial use as biopesticides in the United States (Mahenthiralingam *et al.*, 2005). However, after risk assessment, the United States Environmental Protection Agency placed a moratorium on new registrations of (biopesticidal) products containing these bacteria (Mahenthiralingam *et al.*, 2008). Currently, there is no clear distinction between Bcc isolates originating from an environmental or clinical source, and it is widely accepted that the natural environment is a potential source of Bcc infection (Mahenthiralingam *et al.*, 2008) and that the environmental release of these strains is not without risk.

1.2.3.4 Bioremediators of organic and anthropogenic pollutants

The concept of the natural environmental as a potential source of Bcc infection has greatly impacted the exploitation of Bcc bacteria for other biotechnological uses. Numerous *Burkholderia* strains have been isolated based on their ability to degrade anthropogenic organochemical pollutants; several Bcc have been shown to degrade organophosphorus insecticides, polychlorinated biphenyls (PCBs) and chlorinated ethenes. For example, *B. vietnamiensis* strain G4, a member of the Bcc originally isolated from water with a history of chlorinated solvent pollution, was found to be a potent degrader of the common groundwater contaminant trichloroethylene (TCE) (Nelson *et al.*, 1986). In a field trial test, *B. vietnamiensis* G4 was found to reduce the chlorinated solvent in contaminated aquifers by more than 70% (O'Sullivan & Mahenthiralingam, 2005).

In order to harness beneficial traits such as these, the associated health risks of Bcc bacteria must be significantly reduced or eliminated. The development and potential use of bioengineered strains with attenuated virulence that retain biotechnologically useful traits may be an option to overcoming this problem. The plasticity of the Bcc genome and the multifactorial nature of Bcc pathogenicity may make attenuation by genetic modification difficult but not necessarily impossible (Mahenthiralingam *et al.*, 2008). Alternatively, to avoid all associated health risks, beneficial genetic pathways from Bcc strains could be transferred to host strains that lack the capacity to cause infection. The main difficulties associated with this approach would be replicating the genomic context in which the trait is successfully expressed; important genetic factors, other than the genes or gene pathways themselves, may have to be identified (Denef, 2007). Recent discoveries expanding the potential biotechnological uses of Bcc bacteria to a clinical context (e.g anti-tumour drug (Partida-Martinez, 2005) and novel antibiotic (Mahenthiralingam *et al.*, 2011)), may well encourage the further development of such strategies.

1.2.4 Bcc bacteria as opportunistic pathogens of humans

Bcc bacteria have the capacity to infect a wide range of organisms, from single-celled protozoa to humans (Mahenthiralingam & Vandamme, 2005). Based on the culture collection at Cardiff University, all Bcc groups with the exception of *B. cenocepacia* IIIC have been isolated from infection, with at least one isolate from each group having been derived from a clinical source (Mahenthiralingam *et al.*, 2008). As Bcc bacteria are not carried as commensal organisms by humans, the main sources of infection in vulnerable individuals are considered to be the hospital environment (nosocomally), patient-to-patient transmission, or the natural environment (Baldwin *et al.*, 2007). Several reports have identified the use of contaminated industrial products (e.g. disinfectants, antiseptics, pharmaceuticals) and contaminated medical devices as sources of nosocomial outbreaks of Bcc infection (Mahenthiralingam *et al.*, 2005). In rare cases, contaminated personal care products have been reported as sources of Bcc outbreaks of infection in vulnerable individuals (discussed in detail in section 1.2.5).

Bcc species emerged as significant pathogens of cystic fibrosis (CF) patients over 30 years ago, CF being the most common autosomal recessive hereditary lethal disease of Caucasians, occurring in approximately one in 2500 live births (O'Sullivan, 2009). The CF lung is particularly vulnerable to microbial colonisation and infection due to insufficient clearing of mucus from the airways (Chmiel *et al.*, 2003). The clinical outcome of Bcc infection in CF patients is highly variable due to host and strain factors. However, Bcc infection is commonly associated with poorer prognosis, longer hospital stays and an increased mortality rate (Mahenthiralingam *et al.*, 2005). Chronic infection is exceptionally difficult to eradicate as all Bcc species are highly resistant to antibiotics (Nzula *et al.*, 2002). Isles *et al.* (1984) first described an increasing prevalence of Bcc infection in a Canadian CF clinic, documenting a rapid and fatal deterioration of health in a minority of Bcc infected patients; a clinical course subsequently described as ‘cepacia syndrome’.

In the early 1990s, molecular epidemiological studies confirmed that patient-to-patient transmission of Bcc infection could occur within a hospital environment or through social contact. As a result, Bcc infected CF patients were cohorted and treated separately from patients with *Pseudomonas* infection or patients without infection (Mahenthiralingam *et al.*, 2008). By the mid 1990s, there were several reports of a highly transmissible and highly virulent so called “epidemic” Bcc strain common to multiple patients in the

Canadian and United Kingdom CF population. This strain, initially designated as *B. cenocepacia* electrophoretic type (ET) 12 (Johnson *et al.*, 1994), was presumed to have spread to the UK from Canada via patient-to-patient contact at a CF summer camp (Govan *et al.*, 1993). Several virulence factors have been described for *B. cenocepacia* strains (as reviewed by (Mahenthiralingam *et al.*, 2005). ET-12 strains (also known as cable pilus strain (Sun *et al.*, 1995) or RAPD type II (Mahenthiralingam *et al.*, 1996)) are characterised by a unique combination of two virulence factors in particular: expression of cable pili believed to facilitate adhesion and colonisation of lung epithelial cells, and a pathogenicity island referred to as the '*B. cepacia* epidemic strain marker' (Mahenthiralingam *et al.*, 2005). Subsequent studies have identified additional Bcc CF epidemic strains belonging to the *B. cenocepacia* clonal lineages and other species groups, including *B. multivorans, B. pyrrocinia* and BCC group AT (Coenye & Lipuma, 2007a). The *B. cenocepacia* "Midwest clone" accounts for considerable infection in the Midwestern region of the U.S, and the *B. cenocepacia* PHDC clone has been recovered from the majority of CF patients in the mid-Atlantic region and three European countries. The three *B. cenocepacia* strains (ET12, Midwest and PHDC) account for a large number of infections worldwide and have caused significant morbidity and mortality in the CF community over the past two decades (Mahenthiralingam *et al.*, 2005).

Since its emergence as a problematic CF pathogen, epidemiological surveillance of Bcc species in CF patients has been of paramount importance. All Bcc species, with the exception of *B. ubonensis,* have been isolated from CF sputum (LiPuma, 2010). The prevalence of each Bcc species in the CF lung has also been extensively investigated. Although there may be regional differences present in the epidemiological distribution of Bcc in CF, *B. cenocepacia* is historically considered to be the dominant Bcc pathogen in the CF lung followed by *B. multivorans*, with a mean prevalence of 67 % and 17 % respectively, in CF populations (USA, Canada and Italy), prior to 2002 (Mahenthiralingam *et al.*, 2008). Highly virulent, *B. cenocepacia* has the capability to super-infect and replace infection by *B. multivorans.* However, a recent review of Bcc CF infection in the UK clearly demonstrates a change in epidemiology (Govan *et al.*, 2007), with a reduction in the spread and prevalence of transmissible *B. cenocepacia* strains and a consequential increase in prevalence of *B. multivorans*. Other countries have reported similar epidemiological changes. This phenomenon may be a result of the enforcement of stringent infection control measures or the higher mortality rates associated with *B.*

cenocepacia infection (Drevinek & Mahenthiralingam, 2010). The remaining formally named species account for less than 10 % of all Bcc CF infection, and with the exception of *B. dolosa* strain SLC6 in the U.S, appear to be infrequently shared among CF patients (Coenye & Lipuma, 2007a; Reik *et al.*, 2005). In the U.S., *B. cepacia, B. stabilis, B. vietnamiensis* and *B. dolosa* were more likely to be encountered as CF pathogens than *B. ambifaria, B. anthina* and *B. pyrrocinia* (Reik *et al.*, 2005).

Understanding the clinical epidemiology of Bcc infection outside the CF community is far more challenging as outside of CF the Bcc cause a relatively small number of infections and is often difficult to identify accurately (Mahenthiralingam *et al.*, 2008). Clinical isolates from non-CF patients indicate a disparate distribution of Bcc species from that encountered in CF populations. Reik *et al*. (2005) examined Bcc species diversity in isolates recovered from 1218 CF patients and 90 patients without CF , collected between 1996 and 2004 in the U.S. *B. cenocepacia* (25.6 %) and *B. cepacia* (18.9%) were the most commonly encountered named species groups in non-CF infection; however, "species indeterminate" isolates infected a greater number of the non-CF patients, recovered from approximately 22% of the non-CF patients and 1% of the CF patients. *B. multivorans,* a common CF pathogen, was the fourth most commonly encountered group in non-CF. *B. dolosa* and *B. pyrrocinia,* isolated from 46 (3.8%) and 3 (0.3%) CF patients respectively, were not isolated from non-CF clinical specimens in this study.

Infection control procedures have reduced patient-to-patient spread of highly transmissible strains of Bcc within the CF community but they have not eradicated the emergence of new Bcc infection. Does the natural environment act as a reservoir for infectious Bcc pathogens? In 2002, genetic typing methods revealed that isolates abundant in onion fields of New York State were indistinguishable from the epidemic *B. cenocepacia* strain PHDC (LiPuma *et al.*, 2002). Several studies have since demonstrated further instances of genetic identity between environmental and clinical Bcc isolates (Payne *et al.*, 2005), challenging the historic view that they are somehow distinct from each another. Baldwin *et al*. (2007) used the highly discriminatory typing method MLST to study Bcc isolates from several large bacterial collections. Greater than 20% of the clinical isolates were indistinguishable by MLST from isolates from environmental sources. Clinical isolates belonging to *B. cepacia, B. multivorans, B. cenocepacia recA* lineages IIIA and IIIB, *B. stabilis, B. vietnamiensis* and *B. ambifaria* were indistinguishable at all seven loci from isolates recovered from the natural environment (e.g. river water, onion, radish, the maize

rhizosphere), the hospital environment (e.g. medical equipment), industrial processes (e.g. pharmaceuticals, personal care product) and environmental industrial sources (Baldwin *et al.*, 2007; Mahenthiralingam *et al.*, 2008). The overlap of Bcc sequence types from different isolation sources within the Mahenthiralingam group culture collection at Cardiff University are shown in Figure 2.

1.2.5 Bcc as contaminants of industrial processes

Highly versatile, Bcc bacteria have the ability to survive and potentially proliferate as contaminants of a diverse range of man-made products including anti-infective solutions (e.g. disinfectants and antiseptics), pharmaceuticals, cosmetics (e.g. personal care products) and petroleum products. Bcc contamination of commercial goods can result in an economic loss to the manufacturer, and may pose a risk to the health of vulnerable consumers (as discussed in section 1.1.1.). Individual infection due to Bcc-contaminated industrial product is not documented in significant detail. Therefore, reported Bcc infections are more likely to be nosocomial outbreaks or pseudoepidemics (Lundov *et al.*, 2009).

The Bcc has historically been associated with the contamination of commonly used disinfectants and antiseptic solutions. Contamination of antiseptics and disinfectant solutions by Bcc bacteria may occur during industrial manufacture, via dilution with non-sterile water, or by inappropriate handling (e.g. repeated use of an anti-infective container) (Nasser *et al.*, 2004; Oie & Kamiya, 1996). Since the 1970s, numerous reports have been published on outbreaks of Bcc infection as a result of using contaminated anti-infective solutions directly (e.g. skin antiseptics), or in the sanitation of medical equipment (Anderson *et al.*, 1991; Heo *et al.*, 2008; Romero-Gomez *et al.*, 2008; Weber *et al.*, 2007). The ability of Bcc bacteria to survive prolonged exposure to solutions containing high concentrations of commonly used anti-infectives such as benzalkonium chloride, chlorhexidine gluconate and povidone-iodine, cetylpyridinium chloride and triclosan (Geftic *et al.*, 1979; Rose *et al.*, 2009), may well play an important role in allowing these organisms to cause infection.

The Bcc are recognised as universal contaminants of sterile and non-sterile pharmaceuticals and personal care products (Jimenez, 2001a). Use of contaminated pharmaceuticals such as nebuliser solutions (Balkhy *et al.*, 2005; Ghazal *et al.*, 2006), non-sterile saline (Cunha *et al.*, 2007), multi dose heparin solutions (Yang *et al.*, 2008),

ultrasound gel (Hutchinson *et al.*, 2004; Jacobson *et al.*, 2006) and nasal sprays (Dolan *et al.*, 2005) has been attributed to outbreaks of Bcc infection in hospitalized or vulnerable individuals. Several outbreaks of nosocomial Bcc infection attributed to the use of contaminated personal care products have also been reported. The use of contaminated mouthwash in particular has been associated with the outbreak of Bcc infection in patients who are intubated or have increased risk of aspiration (Matrician *et al.*, 2000). In 2005, a multistate outbreak of *B. cenocepacia* colonisation and infection in patients at increased risk of pneumonia was associated with an intrinsically contaminated alcohol free mouthwash (Kutty *et al.*, 2007). Subsequent evaluation of the product suggested contamination had occurred prior to consumer use and that levels of the preservative agent cetylpyridinium chloride in the product were inadequate.

An outbreak of *B. cepacia* infection in five severely ill patients in an intensive care unit of a Barcelona hospital was associated with intrinsically contaminated moisturising body-milk (Alvarez-Lerma *et al.*, 2008). Pulse-field gel electrophoresis profiles of isolates from patients were indistinguishable from that of isolates recovered from hermetically closed units containing product. Contamination of the moisturiser had presumably occurred during the manufacturing process, transportation or storage stages, before application of the product to the patients by nursing staff (Alvarez-Lerma *et al.*, 2008). Recently, the first nosocomial *B. contaminans* outbreak associated with prefabricated moist washcloths was reported in a German hospital (Martin *et al.*, 2011). Over a five month period, 61 intensive care patients were infected, some with severe infection. The reported outbreaks of Bcc infection were all terminated once the detected source was eliminated. All cases recommended that non-sterile personal care products should only be used with critically ill patients once the associated infection risks have been thoroughly assessed.

A testament to their catabolic versatility, Bcc bacteria are frequently isolated as contaminants in the petroleum industry (O'Sullivan & Mahenthiralingam, 2005). Contamination of hydrocarbon fuels can lead to costly and dangerous operational problems in the storage of fuels and its use in engine systems (White, 2010). White *et al.* (2010) recently described the abundance and diversity of fuel-contaminating bacteria. Systematic analysis of a collection of 152 isolates from 54 fuel samples demonstrated a dominance of *Pseudomonas* (21%) and *Burkholderia* (7%) species. Eight Bcc isolates capable of utilising phenol vapour and a selection of aliphatic hydrocarbons as substrate, were recovered from a single oil refinery site alone. *B. vietnamiensis*, a species with the capacity

to degrade a range of xenobiotic compounds (e.g. toluene, phenols, and trichloroethylene) commonly found in fuels (O'Sullivan & Mahenthiralingam, 2005) appeared to be widespread, and was recovered from multiple unrelated automotive diesel samples (White, 2010).

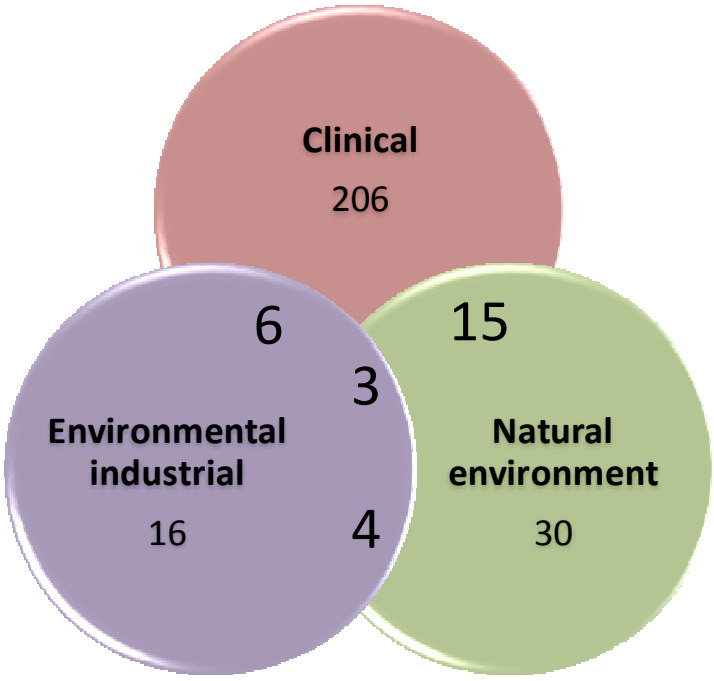


Figure 2. Shared genetic identity of Bcc isolates from clinical, environmental, and environmental sources.

798 Bcc isolates within the Mahenthiralingam group culture collection at Cardiff University were assigned to 376 sequence types (ST) by MLST analysis. The total number of ST and the number of overlapping sequence types between the three isolation sources are shown. By MLST analysis, 30.1% of clinical isolates are indistinguishable from isolates from the natural environment, 12.4% are indistinguishable from environmental industrial isolates. Adapted from Mahenthiralingam *et al*. (2008).

1.3 PREVENTING MICROBIAL CONTAMINATION: PRESERVATION OF PERSONAL CARE PRODUCTS

One of the main principles of preservation is the retardation of growth and/or killing of contaminating microorganisms that may gain access to the process stream, or during consumer use (Orth, 2005). Low levels of chemical biocides, commonly referred to as preservative agents, are often incorporated into raw materials and finished personal care products to protect them from microbial contamination. The selection of preservative agents for use is a complex process involving many factors. These include: whether an agent is regulated for use for a particular country; the formulation conditions e.g. pH or water activity, preservative solubility; the antimicrobial spectrum of an agent; the effect of formulation ingredients on activity, process conditions and packaging effects (Orth *et al.*, 2006). Combinations of preservatives (preservative systems), conferring a wide antimicrobial spectrum, are commonly used in personal care products to protect against bacterial and fungal contamination. In addition, combinations of agents enable adequate preservation using lower concentrations of individual chemicals, thereby, protecting the consumer and limiting the development of microbial resistance to a single agent (Chopra, 1996). Preservative combinations with synergistic activity have additional benefits, providing a level of antimicrobial action beyond that of the additive activity of the individual agents.

1.3.1 Regulation of preservative agents

To protect the consumer from often prolonged or repeated exposure to chemical agents, the preservation of personal care products is strictly regulated. In Europe, the Council of the European Communities formulated the European Economic Community (EEC) Cosmetics directive which states the principles and rules of marketing personal care products (Orth *et al.*, 2006). Attached to the directive 76/768/EEC (www.ec.europa.eu) are annexes which list the preservatives which may or may not be used and stipulates maximum levels of use. Prior to approval for use, preservatives are subjected to a wide review of safety testing to ensure the effective use level is not associated with adverse effects to the consumer (Orth *et al.* 2006). In Japan, a similar system of regulation is applied to the industry. Approved, restricted and not approved agents are published by The Ministry of Health and Welfare as positive and negative lists. Japan has the most restrictions and fewest permitted agents (Orth *et al.*, 2006). Regulation of personal care products in the U.S. takes a different approach. Although there is no official list of

approved preservatives the FDA restricts or prohibits use of agents with associated health risks. In addition, the Cosmetic, Toiletry and Fragrance Association (CFTA) fund the independent review and safety assessment of preservative agents (Orth *et al.*, 2006).

Preservative efficacy testing, commonly referred to as challenge testing, is conducted to determine the minimum effective concentration of one or more preservative and to ensure raw materials and finished products are adequately preserved and capable of withstanding microbial insult during manufacturing and throughout consumer use (Orth *et al.*, 2006). Several standardised methods of challenge testing, published by professional associations, are used in the personal care industry in different countries. These include methods from the US Pharmacopeia, CFTA, European Pharmacopeia and Japanese Pharmacopeia, rapid procedures such as ATP-bioluminescence (Jimenez, 2001b) and linear regression methods (Orth *et al.*, 1998). Test organisms should be representative of those likely to be encountered as contaminants during the manufacturing process and consumer use (Russell, 2003a). Bacteria, yeasts and moulds are used during challenge testing.

For bacterial efficacy evaluations, a range of morphological and physiological types are evaluated, including Gram-positive cocci (*S. aureus* and *Bacillus spp*, if desired), Gram-negative fermenters (*E. coli*) and Gram-negative non-fermenters (*P. aeruginosa* and *B. cepacia*) (Orth *et al.*, 2006). The majority of recommended challenge microorganisms are from national culture collections. In addition, certain methods recommend the inclusion of "in-house" isolates recovered from contaminated earlier batches of a product, raw material or the industrial environment. Test organisms are inoculated, usually as a single challenge (but can be a mixed inocula), into samples of the test product or material to achieve test concentrations ranging from 10^5 to 10^6 CFU/g (representing gross contamination). Aliquots of the sample are removed at specified intervals, neutralised according to protocol and viable CFU quantified usually by plate count methods; data are interpreted according to pharmacopoeial or other official documentation (Russell, 2003a). For example the CFTA acceptance criteria define adequate preservation as a 99.9% reduction in bacterial counts within seven days, and no further increase for the duration of the test period (Orth *et al.*, 2006).

1.3.2 Key preservatives in the personal care industry

Numerous factors influence the frequency at which a preservative agent is utilised within an industry. In the personal care industry, these factors include regulations, cost, spectrum of activity, level of efficacy, toxicity to the consumer, solubility, stability at high process conditions and compatibility of a preservative agent with other product ingredients. The frequency of preservative use is dynamic, adjusting to changes in regulation and consumer pressure. Voluntary disclosure to the FDA by manufacturers in 1993, revealed over 100 preservatives were used in personal care products in the US. Parabens (esters of para-hydroxybenzoic acid) were the most commonly used agents, followed by imidazolidinyl urea (formaldehyde releaser), isothiazolones, quarternium-15 (formaldehyde releaser), formaldehyde, phenoxyethanol and bronopol. In recent years, concerns over the reported weak estrogenic activity of parabens led to a slight decrease in use associated with a consumer demand for 'paraben-free' products (Orth *et al.*, 2006). However, a recent review of preservative use in the US and Canada would suggest the use of parabens is increasing once again, and that cosmetic companies which joined the 'paraben-free' manufacturing practice are now increasingly formulating with them again (Steinberg, 2010). Preservatives on the decline in the US and Canada included Imidazolinyl urea, Quaternum-15 and triclosan (Steinberg, 2010). Stricter regulation of preservatives in EU-regulated countries and Japan means fewer preservative agents can be utilised. However, the frequency of use is similar to the US in that parabens are the most commonly used preservatives and that many formulations utilise formaldehyde releasing agents (Russell, 2004b). Also increasing in frequency is the use of chemicals, not listed as preservatives in the EU and Japan, that offer additional benefits to the formulation, such as enhanced antimicrobial activity. These include caprylyl glycol, ethylhexylglycerin and penetylene glycol.

The following sections discuss the preservatives and chemical agents that form the focus of this study. The chemical structure and maximum EU regulated levels of these agents permitted for use in rinse-off personal care products are shown in Table 2.

1.3.2.1 Parabens and benzoic acids

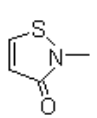
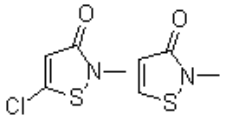
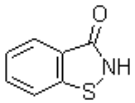
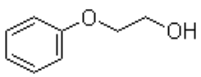
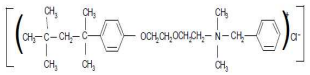
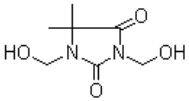
Over the past 75 years, the parabens (methyl, ethyl, butyl and propyl esters of *para*-(4)-hydroxybenzoic acid) have become one of the most commonly used preservative chemicals in cosmetics and pharmaceuticals, due in part to their high efficacy and low toxicity (Hoberman *et al.*, 2008). Parabens are active over a wide pH range (4-8), and have low water solubility which decreases proportionately with increases in alkyl chain length (order methyl to butyl). Parabens and benzoic acids are examples of preservatives that migrate (according to solubility) from the water to the oil phase of emulsions. In order to preserve adequately such a formulation it is essential that the preservative is present in the water phase. Mixtures of esters are commonly used to preserve both the aqueous and oil phases of emulsions and/or chemicals are added to the water phase to decrease the partition coefficient. Parabens are generally more active against Gram-positive bacteria and fungi than Gram-negative bacteria. At high concentrations, parabens exert a predominantly bacteriostatic and fungistatic activity, affecting the cytoplasm/plasma membrane causing leakage of intracellular components (Russell, 2003b). Lower concentrations dissipate the proton motive force and can cause an acidification of the cell cytoplasm (Figure 3) (Maillard, 2002; Russell, 2003b).

Benzoic acid (a lipophilic weak acid) are most effective against Gram-positive and Gram-negative bacteria and yeasts at acidic pH (≤ 5), when in the undissociated form. Salts of benzoic acid such as sodium benzoate are antimicrobial inactive, unless added to the water phase of the formulation, by which free acid is generated (Orth *et al.*, 2006). The antibacterial action of benzoic acids is similar to the parabens, dissipating the proton motive force at the cell membrane and inhibiting active transport (Figure 3) (Russell, 2003b).

1.3.2.2 Formaldehyde-donors

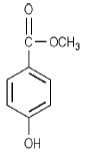
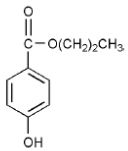
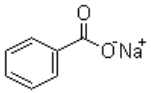
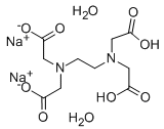
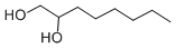
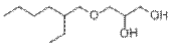
Formaldehyde (FA) is widely used in a variety of settings for disinfection (in liquid and gaseous state), antisepsis and preservation (as formaldehyde solution) purposes (Russell, 2004b). Formaldehyde-donor preservatives [imidazolidynly urea, dimetholdimethyl hydantoin (DMH), diazolidinyl urea, quarternium-15 and bronopol] are commonly utilised in personal care products. The rate of formaldehyde release (upon decomposition of the donor) is largely dependent on pH, with rapid release occurring at alkaline pH (9-10.5) and slower release associated with acidic pH (3-5) (Russell, 2004b). Formaldehyde is an alkylating agent with lethal activity against bacteria and fungi, interacting with proteins, nucleic acid bases of RNA, and to a lesser extent, DNA (Russell, 2004b). Formaldehyde reacts with protein molecules by binding to the primary amide and amino groups, resulting in the formation of intermolecular cross-linkage (Russell, 2004b). Consequently, the presence of amines, amides, or hydrolysed proteins in personal care products may significantly reduce the amount of free-formaldehyde available (Doi *et al.*, 2010).

Table 2. Preservatives and preservative enhancing agents used in this study

Class of agent	Isothiazolinones			Alcohols	QAC	Formaldhyde donor
Preservative [1]	Methylisothiazolinone	chloromethylisothiazolinone & methylisothiazolinone	Benzisothiazolinone	Phenoxyethanol	Benzethonium chloride	Dimethylol dimethyl hydantoin
Trade name	Neolone 950	Kathon CG	Nipacide BIT-20	Phenoxetol	Lonzagard	Glydant
Abbreviation	MIT	CMIT/MIT	BIT	PH	BC	DMH
Supplier	Rohm & Haas	Rohm & Haas	Clariant	Clariant	Lonza	Lonza
Molecular formular	C_4H_5NOS	$C_8H_9ClN_2O_2S_2$	C_7H_5NOS	$C_8H_{10}O_2$	$C_{27}H_{42}ClNO_2$	$C_7H_{12}N_2O_4$
Chemical structure						
Activity (%)[2]	9.7	1.498	20	>99	100	54
Regulated level (%) [3]	0.01	0.0015	0.2 [Ж]	1	0.1	0.3

Footnotes:
[1] INCI designation
[2] Activity of Ingredient(s) as listed on Material Data sheet
[3] Maximum authorised concentrations according to EU cosmetics directive 76/768/EEC, annex VI- list of preservatives which cosmetics may contain.
[β] As a free acid
§ Per ester, 0.8 as total
[Ж] Not permitted for use in EU regulated countries, manufacturers recommended level
Supplier information: Clariant Ltd Horsforth Leeds UK; Lonza Ltd, Basel Switzerland;
Dr Straetmans Chemische Produkte GmbH Germany; Schulke & Mayr, Schulke & Mayr GmbH Norderstedt Germany. Rohm & Haas, Coventry UK; Fisher Scientific Ltd, Loughborough, UK

Table 2. Preservatives and preservative enhancers used in this study (continued)

Class of agent	Parabens and benzoic acid			Preservative enhancer		
Preservative [1]	Methyl Paraben	Propyl Paraben	Sodium Benzoate	Ethylenediamine-tetraacetic acid disodium salt	Caprylyl Glycol	Ethylhexyl Glycerin
Trade name	Nipagin M	Nipasol M	Sodium Benzoate	EDTA	Dermosoft Octiol	Sensiva SC50
Abbreviation	MP	PP	SB	EDTA	CG	EG
Supplier	Clariant	Clariant	Fisher Scientific	Sigma-Aldrich	Dr.Straetmans	Schulke & Mayr
Molecular formular	$C_8H_8O_3$	$C_{10}H_{12}O_3$	$C_7H_5O_2Na$	$C_{10}H_{14}N_2Na_2O_8.2H_2O$	$C_8H_{18}O_2$	$C_{11}H_{24}O_3$
Chemical structure						
Activity (%)[2]	100	100	100	100	100	100
Regulated level (%) [3]	0.4	0.4	0.5	0.1[β]	1	0.5

Footnotes:
[1] INCI designation
[2] Activity of Ingredient(s) as listed on Material Data sheet
[3] Maximum authorised concentrations according to EU cosmetics directive 76/768/EEC, annex VI- list of preservatives which cosmetics may contain.
[β] As a free acid
§ Per ester, 0.8 as total
Ж Not permitted for use in EU regulated countries, manufacturers recommended level
Parabens are esters of para-hydroxybenzoic acid.
Supplier information: Clariant Ltd Horsforth Leeds UK; Lonza Ltd, Basel Switzerland;
Dr Straetmans Chemische Produkte GmbH Germany; Schulke & Mayr, Schulke & Mayr GmbH Norderstedt Germany. Rohm & Haas, Coventry UK; Fisher Scientific Ltd, Loughborough, UK

1.3.2.3 Isothiazolones

Isothiazolone biocides, such as *N*-methylisothiazolinone (MIT), 5-chloro-*N*-methylisothiazolinone (CMIT) and benzisothiazolinone (BIT), are used in a variety of industrial settings (Nicoletti *et al.*, 1993). Commercially available suspensions of these agents are water soluble, biodegradable to non-toxic metabolites and are generally of high compatibility with product ingredients. The broad-spectrum microbistatic activity and stability in a range of pH and temperatures have made methylisothiazolinone a favourable alternative to formaldehyde (Russell, 2004b). Use of chloromethylisothiazolinone and benzisothiazolinone preservatives is more restricted due to irritancy issues. Chloromethylisothiazolinone is only regulated for use in personal care products (not intended to have contact with mucous membranes) as a 3:1 mixture of chloro to non-chlorinated forms. Benzisothiazolinone, currently used in industrial emulsions, adhesives, paints, and household products is not regulated for use in personal care products in EU countries or Japan.

In bacteria, isothiazolone preservatives are thought to react chemically with thiol-containing (e.g. cysteine, glutathione) cytoplasmic and membrane bound enzymes (Denyer, 1995), as their activity is strongly antagonised by thiol-containing materials (Figure 3). The oxidation of thiol groups causes the formation of disulphides which further interact to give thiol dimmers (e.g. cystine, glutathione disulphide) and reduced ring-opened forms of the biocide (Collier *et al.*, 1991). Open-ringed versions of the biocide may then additionally interact with other isothiazolones, resulting in isothiazolone dimmers. In addition, morphological changes associated with bacterial growth in the presence of sub-inhibitory concentrations of chloromethylisothiazolinone would suggest the additional interference and possible inhibition of DNA replication (Chopra, 1996).

1.3.2.4 Alcohols

Several alcohols possess a broad-spectrum antimicrobial activity against bacteria, viruses, and fungi but are not sporacidal (McDonnell & Russell, 1999). As such, alcohols are used extensively for hard surface disinfection and skin antisepsis purposes; ethyl alcohol, isopropyl and *n*-propanol are most widely used (McDonnell & Russell, 1999). Although several alcohols are regulated for use in the personal care industry, the market is dominated by phenoxyethanol. In 2007, phenoxyethanol was the third most used preservative of cosmetics in the US, found in 20% of voluntary registered products (Lundov *et al.*, 2011). 2-phenoxyethanol acts against Gram-negative bacteria but has weak

anti-fungal properties. As such, phenoxyethanol is often used in combination with agents that display strong anti-fungal activity but weaker activity against Gram-negative bacteria (e.g. parabens) (Orth *et al.*, 2006). As a membrane active agent, phenoxyethanol induces generalized loss of cytoplasmic membrane function, the extent of damage being largely dependent on concentration (Figure 3). At low concentrations, phenoxyethanol is an uncoupler of oxidative phosphorylation, resulting in the disruption of the proton motif force (PMF), inhibition of membrane bound enzymes and possibly proton translocation (Denyer, 1995) (Maillard, 2002).

1.3.2.5 Quaternary ammonium compounds (QACs)

Quaternary ammonium compounds are used extensively in both clinical and industrial settings as disinfectants, antiseptics and as preservatives of cosmetics and pharmaceuticals. Benzethonium chloride and benzalkonium chloride are two frequently used QACs in the personal care industry. QACs are primarily active against Gram-negative and Gram positive bacteria however, *P. aeruginosa* tends to be highly resistant (Russell, 2004b). As membrane active agents, these cationic biocides predominantly target the cytoplasmic membrane in bacteria (Salton, 1968). Interaction with the phospholipid components in the membrane causes distortion, induces leakage of intracellular components and results in protoplast lysis under osmotic stress (Figure 3) (McDonnell & Russell, 1999). QACs are also known to damage the outer membrane of Gram-negative bacteria, promoting their own uptake into the periplasmic space, inner membrane and intracellular space. Self-promoted uptake involves the displacement of divalent cations from the lipopolysaccharide (LPS) by the biocide. This interferes with cross-bridging between neighbouring molecules and destabilises the outer membrane, resulting in an increased uptake of the agent (Chopra, 1996).

1.3.2.6 Preservative enhancing agents

Chemical agents not listed as preservatives are increasingly utilised in the personal care industry to enhance the activity of preservative agents. For example, propylene glycol, added to the aqueous phase of an emulsion, can increase the aqueous solubility of lipophillic or oil soluble preservatives (e.g. parabens) that may migrate out of the vulnerable water phase of emulsions. Raw materials and chemicals that enhance activity of preservatives by increasing influx into the microbial cell are also used. EDTA is utilised to increase the antimicrobial activity of a number of preservative agents including QACs, parabens, imidazolidinyl urea, and DMDM hydantoin (Orth *et al.*, 2006). In Gram-negative bacteria, the lipophilic outer membrane is attached to the peptidoglycan cell wall by divalent bridges (Orth *et al.*, 2006), EDTA removes the cation bridges and causes a massive release of LPS (Figure 3). The deficiency of LPS in the outer membrane is believed to be compensated by gylcerophospholipds. As a result, the patches of phospholipid bilayer are more permeable to lipophilic compounds, and there is a greater influx of antimicrobial agents into the cell (Bolla *et al.*, 2011). Ethylhexylglcerin is an emollient, solvent and fixative with antimicrobial properties. Increasingly used in combination with alcohols, it is thought that ethylhexylglycerin enhances their antimicrobial activity by increasing uptake of the preservative into the microbial cell (Gaonkar *et al.*, 2006). Caprylic/capric acid glycerides (a mixture of mono,di and triglycerides) are emollients derived from coconut. These provide a natural bacteriostatic activity, but can also increase the antimicrobial activity of paraben preservatives (Orth *et al.*, 2006).

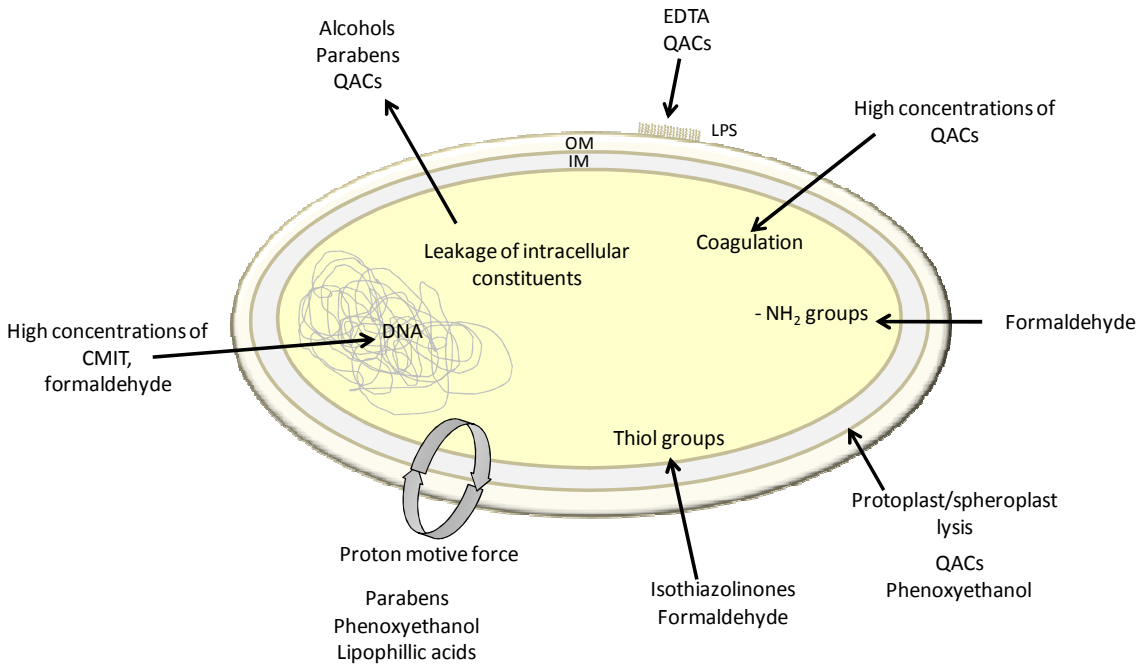


Figure 3. The multiple target sites of preservative agents.

The schematic, to be used in conjunction with relevant text sections 1.3.2, summarises the multiple and concentration-dependent target sites (for Gram-negative bacteria) of key preservatives and a preservative enhancing agent used in the personal care industry that are featured in this study. OM, outer membrane; IM, inner membrane (or cytoplasmic membrane); LPS, lipopolysaccharide; QACs, quaternary ammonium compounds; EDTA, Ethylenediaminetetraacetic acid. Adapted from Russell and Chopra 1996; Maillard, 2002.

1.4 BACTERIAL RESISTANCE TO PRESERVATIVES

Preservatives have been utilised in sterile and non-sterile products for many years and the development of resistance is not a new phenomenon. Resistance to QACs for example, introduced as a disinfectant/preservative in the mid-1930s, was first reported 60 years ago (Chaplin, 1952). There are numerous reports in the literature of bacteria developing resistance to a range of preservatives with diverse chemistries that are used in the personal care industry (Table 3). Early concerns expressed over 20-30 years ago relating to the development of preservative resistance are perhaps more relevant in recent years, as the use of biocides in clinical industrial and domiciliary settings increases while the rate at which new biocides are being introduced decreases. Understanding the interaction between bacteria and preservatives and the development of resistance is therefore essential, in order to:

(1) reduce the economic loss associated with contamination of preserved industrial processes;
(2) protect the consumer;
(3) efficiently treat resistant organisms; and
(4) facilitate strategies to circumvent resistance thus maintaining the efficacy and range of preservatives regulated for use in the personal care industry.

Bacterial mechanisms of resistance can either be a natural property of an organism (intrinsic) or acquired through mutation or by acquisition of plasmids or transposons (chromosomal or plasmid integrating) (McDonnell & Russell, 1999). The following sections discuss resistance mechanisms of Gram-negative bacteria with particular emphasis on Bcc bacteria. The latter are highly resistant to antibiotics and biocides (e.g. disinfectants, antiseptics and preservatives), an aspect of their biology that no doubt contributes to their success as opportunistic pathogens and contaminants of industrial processes.

Table 3. Examples of biocides employed as preservatives to which bacterial resistance has been reported

Benzalkonium chloride	Mercuric salts
Benzisothiazolone[1]	Methylisothiazolone
Benzoic acid	Chloro-methylisothiazolone
Chlorhexidine	Methyl paraben
Chlorophenol	Phenoxyethanol
Dibromodicyanobutane	Phenyethyl alcohol
Dimethoxy dimethyl hydantoin	Phenylmercuric acetate
Formaldehyde	Propyl paraben
Glutaraldehyde	Quarternary ammonium compounds
Hydrogen peroxide	Sorbic acid
Imidazolidyl urea	Trifluoromethyl dichlorocarbanilide
Iodopropynyl butylcarbamate	

Footnotes:
Preservative agents shown are permitted for use in personal care products according to regulations unless otherwise stated. [1] not permitted for use in personal care products in EU-regulated countries. Adapted from Chapman (1998) and English (2006).

1.4.1 Intrinsic resistance mechanisms

1.4.1.1 Cellular impermeability and the Gram-negative outer membrane

Gram-negative bacteria are generally more resistant to antimicrobials than Gram-positive bacteria (McDonnell & Russell, 1999), primarily because of the multi-component barrier system of the cell wall. The outer cell layer of Gram-negative bacteria consists of an outer membrane (envelope), a thin peptidoglycan layer, periplasmic space and an inner (cytoplasmic) membrane (Maillard, 2002). The structure of the cellular membranes enables greater control of the ingress and egress of small molecules, including antimicrobials, while the periplasmic space facilitates enzymatic inactivation or modification of antimicrobials (Burns, 2007). The outer membrane is a bilayer, the outer leaflet composed of phospholipid and lipopolysaccharides (LPS), while the inner leaflet is phospholipid in nature. Lipoproteins, porins that form hydrophilic channels, and efflux pump components are also embedded in the outer membrane. The simpler cytoplasmic membrane, a phospholipid bilayer, may also contain proteins (e.g. components of efflux pumps) that may impact on cellular permeability. The phenomenon of cellular impermeability is of particular importance to biocide resistance, as the primary targets of many biocides are often situated within the cell and/or at the cytoplasmic membrane (Maillard, 2002). Therefore, in order for an antimicrobial to elicit a detrimental effect on a cell it must first penetrate the cell and reach sufficiently high enough concentrations. Changes to the LPS, porins or efflux pumps can have a dramatic effect on permeability and consequently susceptibility to antimicrobials.

1.4.1.2 Lipopolysaccharides (LPS) and its unique structure in Bcc bacteria

The LPS of Gram-negative bacteria consists of three covalently linked regions, *viz*:

(i) lipid A, a hydrophobic phosphorylated glucosamine dissacharide with a number of attached fatty acids;

(ii) the core polysaccharide, a complex oligosaccharide attached to lipid A by 3-deoxy-D-manno-2-octulosonate (KDO);

(iii) the outer O-polysaccharide, a chain of repeating units of oligosaccharides (Denyer & Maillard, 2002).

An intact LPS severely hinders the passage of hydrophobic molecules (including several antimicrobials) into the cell interior, by shielding phospholipids. Mutants lacking the O-specific side chain and most of the core polysaccharide, as well as EDTA-treated cells, are significantly more susceptible to hydrophobic antimicrobial compounds (Chopra, 1996).

Modifications to the LPS of Bcc bacteria are likely to serve as the basis for intrinsic resistance to cationic compounds. The core polysaccharide of Bcc bacteria LPS contains less phosphate and 3-deox-D-manno-octo-2-ulsonic acid than other Gram-negative bacteria. In addition, 4 amino-4 deoxyarabinose (Ara4N) residues are attached to the glucosamine dissacharide of the lipid A region. The high content of phosphate-linked arabinose and fewer KDO in the LPS neutralise the negative charge of the outer membrane groups, consequently decreasing the affinity of the outer membrane for cationic and polycationic molecules such as polymyxin antibiotics and quaternary ammonium compounds (QACs) (Burns, 2007; McDonnell & Russell, 1999). The generalised structure of the Gram-negative LPS in contrast to the modified LPS of Bcc bacteria is shown in Figure 4.

1.4.1.3 Porins

Gram-negative bacteria regulate the intracellular ingress and egress of small molecules by hydrophilic channels known as porins (Hancock, 1987). Porins can be separated into two classes depending on their function:

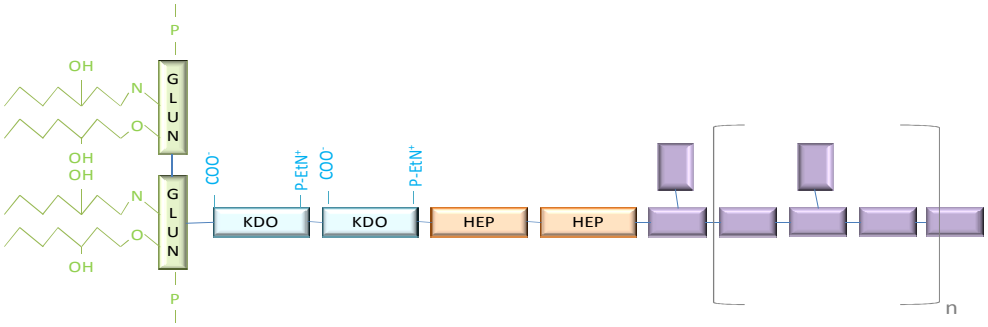
(i) the general diffusion porins that are non-specific;

(ii) specific porins that mediate entry of a specific solute that may not enter the cell via non-specific porin channels (e.g. ferric iron chelates, maltose, nucleosides and vitamin B12)(Denyer & Maillard, 2002) .

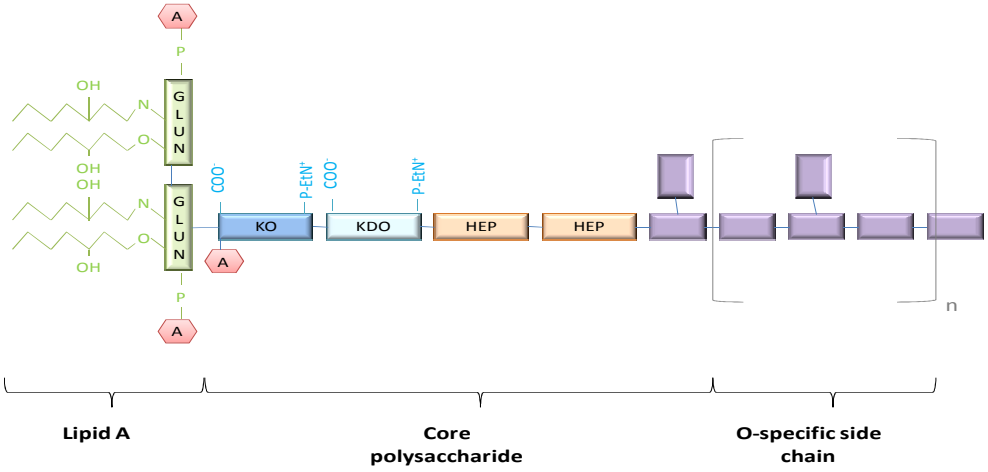
Decreased permeability associated with the alteration of porin size and/or decreased expression of porin proteins has been shown to enhance bacterial resistance to antibiotics and biocides (Denyer & Maillard, 2002). For example, the absence of the outer membrane (porin) protein OprD in *P. aeruginosa* isolates has been associated with a decreased susceptibility to isothiazolone biocides (Brozel & Cloete, 1994; Chapman, 1998; Winder *et al.*, 2000). Similarly, a benzisothiazolone resistant *B. cepacia* isolate recovered from an industrial process had a different outer membrane profile from that of the type strain (Chapman *et al.*, 1998). However, in light of recent taxonomic changes, it is feasible that the isolate may not belong within the *B. cepacia* species group, thus impacting on the credibility of protein profile comparisons with the type strain used. Although porin deficiency in Gram-negative bacteria has been linked with decreased susceptibility to antibiotics and biocides, it may only play a supporting role in the emergence of resistant

organisms. Other mechanisms, such as the efflux or the enzymatic inactivation of antimicrobials, or indeed a combination of factors (e.g. efflux and porin deficiency), are considered more likely to play a significant role (Denyer & Maillard, 2002).

a) Typical LPS of Gram-negative bacteria



b) Modified LPS of Bcc bacteria



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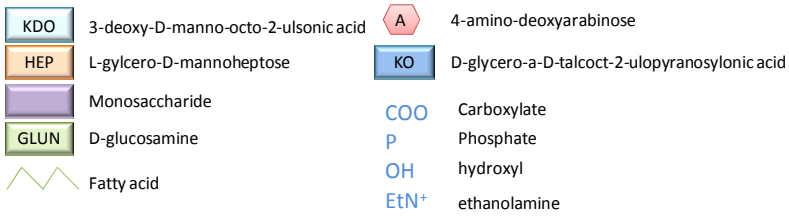


Figure 4. Generalised structure of the lipopolysaccharide (LPS) of Gram-negative bacteria and the modified LPS of Bcc bacteria.

The core polysaccharide of the Bcc LPS (Panel B) contains less 3-deoxy-D-manno-octo-2-ulsonic acid than LPS from other Gram-negative bacteria (Panel A) and 4-amino-deoxyarabinose residues are attached to the glucosamine disaccharide of the lipid A region. Modifications to the Bcc LPS result in a neutralisation of the negative charge of the outer membrane and consequently decrease affinity for binding cationic antimicrobials. Adapted from Russell and Chopra (1996), Cox *et al*. (1991) and Caroff & Karibian (2003).

1.4.1.4 Efflux

The innate protection of the Gram-negative outer membrane and any additional decrease in cellular permeability by the loss of porins cannot prevent the toxic effects of antimicrobials once they have successfully entered the cell. It is becoming increasingly evident that higher levels of antimicrobial resistance in many bacteria are dependent on the constitutive or inducible expression of active efflux systems (Perrin *et al.*, 2010). The induction of efflux as an adaptive response to the presence of an antimicrobial is discussed in section 1.4.3. Efflux systems can be specific for one substrate or can transport structurally unrelated molecules, including antibiotics and biocides of different chemical classes (Piddock, 2006). There are five families of efflux-proteins capable of accommodating multiple antimicrobials, *viz* :

(i) the ATP binding cassette (ABC) superfamily;
(ii) the major facilitator superfamily (MFS);
(iii) the multidrug and toxic-compound extrusion (MATE) family;
(iv) the small multidrug resistance (SMR) family
(a member of the larger drug/metabolite transporter superfamiliy);
(v) the resistance nodulating division (RND) family (Piddock, 2006).

All systems apart from the ABC family (whose members utilise ATP hydrolysis to drive efflux) utilise the proton motive force (PMF) as an energy source to bind substrates from the periplasm and/or cytoplasm and extrude them out of the cell to the external environment (Li & Nikaido, 2004). Consequently, all systems except members of the ABC family function as secondary transporters catalysing antimicrobial-ion (H^+ or Na^+) antiport (Poole, 2004). Examples of the organisation, cellular localisation and substrate for efflux pumps from each family in Gram-negative bacteria are shown in Figure 5.

Efflux has been implicated in resistance to numerous antibiotics including β-lactams, quinolones, tetracycline, chloramphenicol, trimethoprim, sulfamethoxazole and aminoglycosides (Poole, 2005) in Gram-negative bacteria. Efflux-mediated resistance to biocides, employed as anti-infective and preservative agents (e.g. QACs and chlorhexidine), has also been reported (Poole, 2005). For example, chromosomal encoded efflux systems (e.g. *emr* (Lomovskaya & Lewis, 1992)), and transmissible plasmid encoded efflux systems (e.g. *qac*E and *qacE*Δ*1* genes (Paulsen *et al.*, 1993), see section 1.4.2.2) have been associated with QAC resistance in Gram-negative bacteria. Many of the multidrug transporters reported to contribute to QAC resistance belong to the MATE or

RND efflux families. RND efflux systems have also been implicated in resistance to other biocides commonly used in household and personal care products, such as triclosan (Poole, 2005) and chlorhexidine (Coenye *et al.*, 2011).

Only a few multidrug efflux systems from the MFS (Wigfield *et al.*, 2002), MATE (Fehlner-Gardiner & Valvano, 2002) and RND (Guglierame *et al.*, 2006) families have been described in Bcc species. Efflux systems belonging to the RND family are of particular interest as they are associated with significant resistance to drugs and multidrug resistance (Piddock, 2006). Nair *et al.* (2004) first described an RND efflux system in *B. cenocepacia* induced by salicylate that mediated resistance to chloramphenicol, trimethoprim and ciprofloxacin antibiotics. Although homologous to the well characterised Mex-AB-OprM RND system of *P. aeruginosa,* the CeoAB-OpcM operon of *B. cenocepacia* exhibited unique features including a gene encoding a lipase-like protein within its operon (Nair *et al.*, 2004). Subsequent studies have since identified 16 potential RND systems within the *B. cenocepacia* genome (Guglierame *et al.*, 2006; Holden *et al.*, 2009) and 245 putative RND proteins in the 21 completely sequenced *Burkholderia* genomes. Research continues to systematically investigate the involvement of RND efflux in antimicrobial resistance (Bazzini *et al.*, 2011; Perrin *et al.*, 2010).

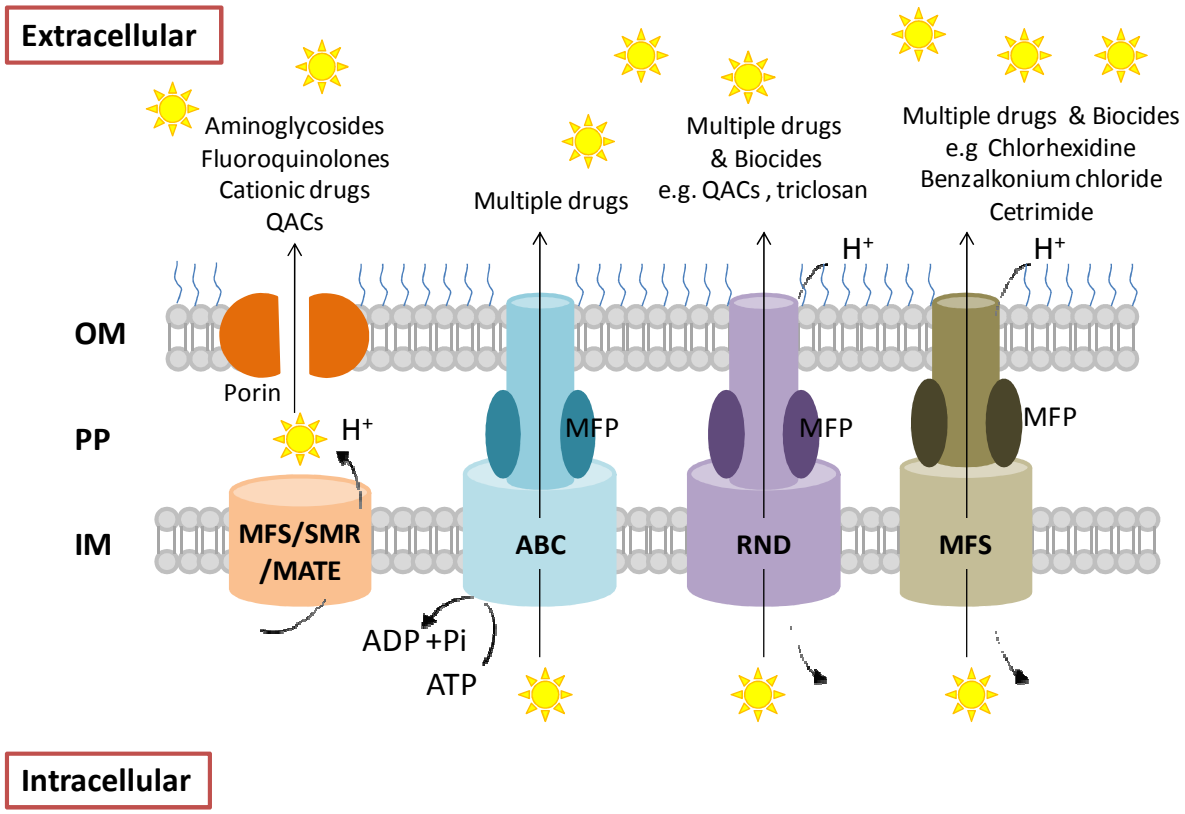


Figure 5. Multi-drug resistance efflux pumps of Gram-negative bacteria.

A schematic diagram of the localisation and structure of representative efflux pump systems from the five main families associated with multi drug resistance: the ATP binding cassette (ABC) superfamily, major facilitator superfamily (MFS), multidrug and toxic-compound extrusion (MATE) family, small multidrug resistance (SMR) family and the resistance nodulating division (RND) family. Substrate specificity may vary according to pump and bacterial species as described by Poole *et al*. (2005), examples of antibiotic and biocide substrates are given. Efflux pumps can be localised at the cytoplasmic membrane or span the cell envelope encompassing the periplasmic space. RND efflux systems typically operate as part of a tripartite system that includes a transporter (efflux) protein (e.g. AcrB) which is located in the inner membrane, a periplasmic fusion protein (membrane fusion protein) (e.g. AcrA) and an outer membrane protein (typically a TolC) which is located in the outer membrane; this organisation is also observed on occasion with ABC and MFS efflux systems. Schematic adapted from Poole *et al.*(2004; 2005) and Piddock (2006). OM, outer membrane; PP, periplasm; IM, inner membrane; MFP, membrane fusion protein.

1.4.1.5 Inactivation or modification

In Gram-negative bacteria, β-lactams and aminoglycoside antibiotics are most commonly associated with enzymatic modification and inactivation (Burns, 2007). Enzymatic modification is rarely the sole resistance mechanism reported in Bcc bacteria. As it is specific to the targeted class of antimicrobial, it is not associated with cross-resistance to unrelated compounds. Inducible β-lactamases, that may hydrolyse and inactivate sensitive penicillins and cephalosporins, have been reported and characterised in the Bcc (Burns, 2007). While Bcc bacteria characteristically possess a high intrinsic resistance to aminoglycosides, the presence of aminoglycoside-inactivating enzymes has not been determined experimentally. However, genes encoding putative aminoglycoside o-phosphotransferase and o-adenyltransferase have been discovered in the *B. cenocepacia* genome (Holden *et al.*, 2009). Expression of the *B. cenocepacia* gene BCAL1756, containing nucleotidyltransferase and phosphotransferase motifs that could possibly confer similar enzyme activities as those present in aminoglycoside-modifying enzymes (Mingeot-Leclercq *et al.*, 1999), was found to be induced in the presence of amikacin (Sass *et al.*, 2011).

Although not considered a major intrinsic mechanism of biocide resistance, several types of biocides (at sub-inhibitory concentration) are readily metabolised by bacteria. These include QACs, benzoic acid, parabens, phenols, chlorhexidine, phenylethanol and formaldehyde (Chopra, 1996; Russell, 2004b). Contamination of personal care products and pharmaceuticals has on occasion been attributed to the bacterial degradation of preservatives (Orth *et al.*, 2006). Bacterial resistance to formaldehyde (FA) and formaldehyde-releasing preservatives mediated by formaldehyde dehydrogenase enzymes is well characterised. Formaldehyde is a highly toxic (aldehyde) intermediate of many biochemical pathways and many organisms possess the ability to detoxify formaldehyde to a non-toxic metabolite (Marx *et al.*, 2004). Increased synthesis of the enzyme has been associated with resistance to this class of biocide (Orth *et al.*, 2006).

FA resistance has been attributed to formaldehyde dehydrogenase genes located on the chromosome of species such as *P. aeruginosa* (Wollmann & Kaulfers, 1991), and self-transmissible plasmids of resistant Enterobacteriaceae (Section 1.4.2.2) (Kaulfers & Brandt, 1987). Four different pathways for the metabolism of formaldehyde are known in bacteria and the genome of *B. cenocepacia* is predicted to contain two of the four pathways (Marx *et al.*, 2004). Bacterial hydrolysis of parabens (*p*-hydroxybenzoic acid) is

also a significant cause of medical and economic concern. Various bacterial species including members of the Bcc have demonstrated the ability to utilise parabens for growth (Amin *et al.*, 2010; Beveridge & Hart, 1970; Hugo, 2001). Increased resistance to parabens has been attributed to the hydrolysis of esters by esterase enzymes in *B. cepacia* and *Enterbacter sp.*(Close & Nielsen, 1976; Valkova *et al.*, 2002).

1.4.1.6 Biofilm formation

Biofilms are described as the growth of surface-associated cells with the production of extracellular polymeric substances (Morton *et al.*, 1998). Bacterial biofilms are dynamically complex, structured biological systems that occur both naturally and in man-made environments and are considered to be the preferred and predominant growth state of many bacteria in nature (Costerton, 1999). Surface-association and biofilm-formation offer a degree of stability in turbulent environments (e.g. rivers, streams); localise cells in close proximity, thus enabling interaction; and offer protection within the growth environment (e.g. against dehydration, UV exposure, salinity and antimicrobial agents) (Hall-Stoodley *et al.*, 2004).

There is a now a large body of work suggesting biofilm associated cells are less susceptible to antibiotics and biocides than their planktonic counterparts (Donlan & Costerton, 2002). Consequently, biofilm-mediated resistance has serious implications in many clinical settings (e.g. persistent infection, device-related infection), as well as environmental and industrial situations (e.g. biofouling, corrosion, persistent & reoccurring spoilage) (Cloete, 2003; Morton *et al.*, 1998). Factors contributing to biofilm associated resistance include:

(i) limited diffusion of an antimicrobial through the exopolysaccharide (EPS) matrix;
(ii) the interaction of antimicrobials with EPS (e.g. the neutralisation or binding of molecules);
(iii) enzyme mediated resistance;
(iv) an altered metabolic state and slow growth rate and of sessile cells within the biofilm due to nutrient and/or electron donor limitations;
(v) the presence of subpopulations of persister cells;
(vi) genetic adaptation (Cloete, 2003; Keren *et al.*, 2004; Mah & O'Toole, 2001).

The tendency to form a biofilm is influenced by numerous environmental signals (see (Karatan & Watnick, 2009)). The process of biofilm formation is considered to proceed through a series of temporal stages from initial attachment to maturation as shown in Figure 6. In recent years, the regulatory networks involved in bacterial biofilm formation, development and dispersal have been the subject of intense study (Karatan & Watnick, 2009). Cyclic diguanosine monophosphate (ci-di-GMP), a ubiquitous secondary signalling molecule widely used by bacteria, has been established as the central regulator of biofilm formation and the main switch from a planktonic to sessile growth state in Gram-negative bacteria (Simm *et al.*, 2004). In general, high intracellular levels of ci-di-GMP have been shown to promote sessility. Two-component regulatory systems (TCS) are a commonly used mechanism by which bacteria process and respond to environmental stimuli. It is therefore unsurprising a large number of TCSs have been reported to be involved in the formation of bacterial biofilms. In their simplest form, a sensor histidine kinase detects environmental cues (directly or indirectly) and elicts an appropriate regulatory response via the transfer of a phosphoryl group to a response regulator. For example, the conserved GacS-GacA system has been shown to regulate a multitude of genes including those involved in exopolysachharide production and biofilm formation in *P. aeruginosa* by two small regulatory RNAs (Karatan & Watnick, 2009).

Quorum-sensing (QS) circuits have been implicated in the regulation of biofilm processes (e.g. formation, development, dispersal and architecture) of several bacterial species (Karatan & Watnick, 2009). QS molecules are small molecules called autoinducers that allow bacteria to co-ordinate their gene expression in a dell density-depended manner; QS circuits activate when extracellular concentrations of an autoinducer exceed that of a certain threshold. The typical QS of Gram-negative bacteria consists of three components: a LuxI synthase homolog, acyl-homoserine-lactone (AHL) signalling molecules and a LuxR receptor homolog (Fuqua & Greenberg, 2002). Several studies have described the effects of mutations within QS related genes and the use of QS inhibitors (or degradation of AHLs) on the formation, maturation and stability of bacterial biofilms (Brackman *et al.*, 2009; Karatan & Watnick, 2009; Wopperer *et al.*, 2006). Studies demonstrate that depending on the species and environmental conditions, QS systems can have a positive or negative effect on biofilm formation (Karatan & Watnick, 2009).

There are numerous reports of Bcc bacteria efficiently colonising and forming biofilms on biotic and abiotic surfaces including roots, glass, sand grains, quartz and metal oxides and plastics (Riedel & Eberl, 2007). It is well established that biofilm-associated Bcc bacteria are less susceptible to antibiotics and biocides compared to their planktonic counterparts (Caraher *et al.*, 2006; Coenye *et al.*, 2011; Desai *et al.*, 1998; Rose *et al.*, 2009). Over the past decade, research has attempted to decipher key factors, genes and regulatory systems involved in the Bcc biofilm process from initial attachment to maturation (summarised in Figure 6). Bcc bacteria possess five different types of fimbriae (pili) that are expressed on the surface of the cell: mesh, filamentous, spine, spike and/or cable (Riedel & Eberl, 2007). With the exception of the cable pili, the role of other pili in initial surface attachment and colonisation remains to be determined. Several studies have focused on the later developmental stages of the Bcc biofilm and exopolysaccharide production. Huber *et al.*(2002) conducted a simple screen of a transposon mutant library to identify mutants incapable of biofilm maturation. It became apparent that several classes of gene were required for the maturation of the *B. cenocepacia* H111 biofilm including genes encoding surface proteins or regulatory factors, genes involved with the biogenesis or maintenance of the outer membrane and cell-to-cell communication systems (e.g. QS systems such as CepI/R).

All *Burkholderia* species utilise QS systems that rely on *N*-acyl-homoserine lactone (AHL) signal molecules to regulate and express certain phenotypic traits in a cell-density dependent manner (Eberl, 2006). Many Bcc strains only possess the well characterised CepI/R system, which relies on C8-HSL, other strains such as *B. vietnamiensis* have additional LuxI/R homologs (e.g. BviI/R) that potentially interact with other systems forming networks (Eberl, 2006; Riedel, 2007). AHL QS mutants of Bcc bacteria demonstrate defective biofilm maturation, forming biofilms with a drastically altered structure and fail to maintain cells within the biofilm (Huber *et al.*, 2001; Tomlin *et al.*, 2005). Phenotypic characterisation of CepI/R system mutants of Bcc strains revealed that in addition to biofilm maturation the system controls the expression of at least 55 different proteins and regulates the expression of swarming motility (flagella-driven movement in the presence of extracellular slime), chitinases, several extracellular enzymes and is required for full virulence in various animal models (Eberl, 2006; Huber *et al.*, 2001; Lewenza *et al.*, 1999; Riedel *et al.*, 2003). Besides the AHL system, several *Burkholderia* species have been shown to utilise a quinolone-dependent QS system (Vial *et al.*, 2008); a communication system first described in *P. aeruginosa* that makes use of hydroxy-alkylquinolines (HAQ) as signal molecules (Heeb *et al.*, 2011).

The production of exopolysaccharide (EPS) by Bcc bacteria has been extensively studied in recent years. Bcc bacteria are found to produce five types of EPS, the majority of strains produce large amounts of an EPS called cepacian (a heteropolysaccharide with a heptasaccharide basic unit) (Goldberg, 2007). Cepacian production plays an important role in biofilm maturation (e.g establishing thick biofilms) and is also considered to play a significant role in persistent colonisation in the CF lung (Riedel & Eberl, 2007).

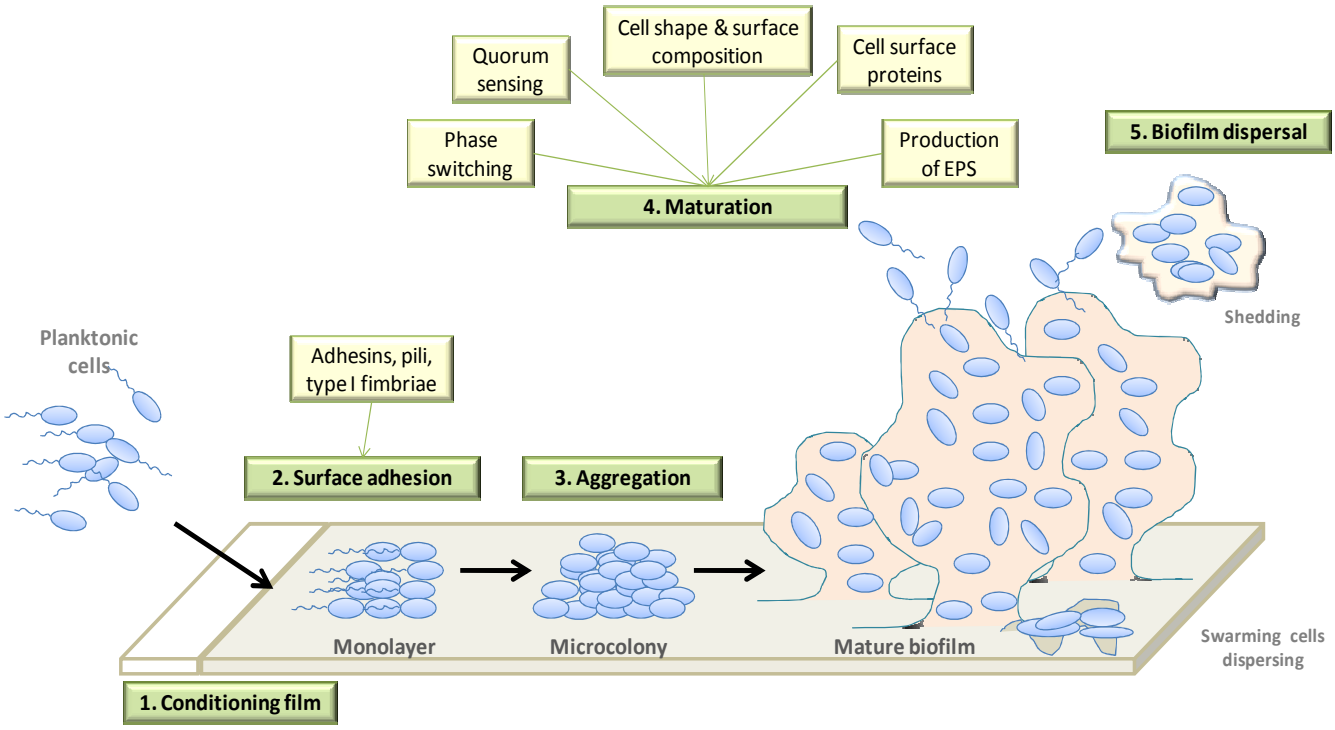


Figure 6. A model of Bcc biofilm formation and development on an abiotic surface.

The five stages of the biofilm process and genes required (shown in yellow): (1) the deposition of organic and inorganic substances on a surface forms a conditioning film; (2) planktonic cells swim towards the substratum using flagella, form a reversible attachment to the surface and migrate to form microcolonies; (3) aggregated cells transition to a non-motile state and form irreversible attachments to the surface; (4) microcolonies differentiate into mature biofilms where cells are embedded in an exopolysaccharide matrix; (5) in the final stages of the biofilm life cycle, aggregates of the mature biofilm may break away and disperse or individual cells may be shed into the external environment. Adapted from Riedel and Eberl (2007).

1.4.2 Acquired mechanisms

In addition to possessing intrinsic resistance to antimicrobials, bacteria can acquire resistance traits through the acquisition of resistance genes, often encoded on mobile genetic elements such as plasmids or transposons (Russell, 1997); via mutation (McDonnell & Russell, 1999); or by amplification of chromosomally encoded resistance genes (Sandegren & Andersson, 2009).

1.4.2.1 Target modification

In contrast to antibiotics, which are generally considered to have specific targets within the bacterial cell, biocides (with the exception of triclosan) are multi-factorial and target multiple cellular components in a concentration-dependent manner. Consequently, site-specific target modification or mutation is not a mechanism often associated with biocide resistance (McDonnell & Russell, 1999). Mutation or modification of the gene encoding the specific target of triclosan (e.g. the FabI enzyme) is one exception that has been observed. For example, in *E. coli* triclosan resistance has been associated with mutations within the *fabI* gene encoding the enoyl-acyl carrier protein reductase of fatty acid biosynthesis (Russell, 2004b).

1.4.2.2 Plasmid mediated

Plasmid-encoded bacterial resistance to antibiotics such as β-lactams, macrolides, chloramphenicol and tetracycline is a well documented phenomenon that often translates into a serious clinical problem (Russell, 1997). The presence of plasmids has also been associated with increased tolerance to biocides including chlorhexidine, QACs, triclosan and formaldehyde (McDonnell & Russell, 1999). In contrast to antibiotic resistance, where acquired resistance has significant clinical importance, the role of plasmids in conferring resistance to biocides is potentially less significant than that of intrinsic mechanisms.

Plasmid-mediated resistance to QACs by virtue of proton-dependent efflux systems has been shown to occur in both Gram-negative (e.g. *qacEΔ1,qacE/F* genes) and Gram-positive bacteria (e.g. *qacA/B, smr, qacG* and *qacH* genes) (Chapman, 2003b). Often located on mobile genetic elements such as conjugative plasmids or intergrons (that commonly carry additional resistance determinants), QAC resistance has the potential to spread rapidly through a population via horizontal gene transfer (Chapman, 2003a) (Russell, 2004b). Reductions in QAC susceptibility mediated by these efflux systems are not large but are still considered to be significant (Chapman, 2003b).

Bacterial resistance mechanisms to the biocide formaldehyde may also be plasmid-encoded (Chapman, 2003a; McDonnell & Russell, 1999; Russell, 2004b). For example, plasmid-encoded changes to the outer membrane proteins of the cell envelope of *E. coli* and *Serratia marcescens* have been associated with a decreased susceptibility to formaldehyde (Kaulfers & Brandt, 1987). Kuemmerle *et al.* (1996) demonstrated that formaldehyde resistance in a clinical isolate of *E. coli* VU3695 was associated with the presence of the gluthione-dependent formaldehyde dehydrogenase (GSH-FDH) gene, *adhC,* located on the large transmissible plasmid pVU3695. Curing of this plasmid significantly decreased production of the protein but failed to abolish GSH-FDH activity altogether, indicating in this instance that the strain possessed an additional (chromosomally encoded) copy or copies of the gene (Dorsey & Actis, 2004).

1.4.3 Adaptive resistance

In contrast to intrinsic and acquired mechanisms of resistance, the phenomenon of adaptive resistance is relatively poorly understood, and until recent years has not been thought to participate in the acquisition of resistance traits and breakthrough of resistant organisms. Both intrinsic and acquired mechanisms of resistance can be characterised as an irreversible phenotype independent of the presence of an antimicrobial and/or environmental stimuli surrounding an organism (Fernández *et al.*, 2011). Adaptive resistance can be characterised as the induction of resistance to one or more antimicrobial agents in response to a specific signal such as the presence of an antibiotic or biocide , an environmental cue (e.g. pH, anaerobiosis) and/or social activities (e.g. swarming motility, biofilm formation) (Fernández *et al.*, 2011; Russell, 2004a). Historically adaptive resistance has been considered transient, with resistance reverting to wild-type levels upon the removal of the specific stimuli; however, there are instances where original levels of susceptibility may not be fully restored (Braoudaki & Hilton, 2004; Mawer & Greenwood, 1978). Several studies now suggest that the level of increased resistance and the duration it persists (stability) is dependent on various factors including the type and dose of antimicrobial, the length of exposure and the bacterial species (Fernández *et al.*, 2011; Russell, 2004a).

Adaptive resistance mechanisms of Gram-negative bacteria are still somewhat unexplored, with most studies corresponding only to a few organisms. Although limited, these studies suggest the mechanisms leading to adaptive resistance are more complex than initially thought, transcriptomic studies demonstrating the involvement of intricate regulatory

systems (Fernández *et al.*, 2011).The major known mechanisms leading to adaptive resistance are diverse, ranging from the induction of specific mechanisms such as degradative enzymes (e.g. inducible class C β-lactamase, formaldehyde dehydrogenase) to non-specific mechanisms such as outer membrane changes and active efflux. As a result, resistance to the same class of antimicrobial and/or cross-resistance to unrelated antimicrobials are phenomena commonly encountered in experiments of adaptation to antibiotics and biocides.

The development of adaptive resistance via exposure to sub-inhibitory concentrations of antibiotics has been well documented in numerous bacterial species over the years. The affect of adaptive resistance on the major antibiotic families (aminogylcosides, fluoroquinolones, β-lactams and polymxyins) and the specific mechanisms involved are reviewed by Fernandez *et al.* (2011). Adaptive resistance to aminogylcosides in particular has been extensively studied, and is perhaps best described for *P. aeruginosa.* Although the mechanisms involved are not fully understood, recent studies demonstrate that reduced intracellular levels of the drug following pre-exposure can be attributed to increased efflux through the MexXY-OprM pump. In addition, pre-exposure to aminoglycosides has been shown to induce the expression of genes involved in the anaerobic respiratory pathway (e.g. *denA* and *anr*). The deviation of cellular energetics to an anaerobic route was also considered to contribute to resistance (Karlowsky *et al.*, 1997) as the accumulation of aminoglycosides depends on a functional respiratory pathway (Taber *et al.*, 1987) in addition to the PMF.

Studies show that the induction of multiple resistance determinants is not an unusual response to the presence of an antimicrobial; for example, multiple resistance mechanisms have been associated with high level resistance to fluoroquinolones in *P. aeruginosa*. Recent microarray studies demonstrate the exposure of *P. aeruginosa* to sub-lethal concentrations of ciprofloxacin (a fluoroquionolone which targets DNA gyrase) results in the altered expression of genes encoding proteins from various functional groups including the overexpression of genes encoding the efflux system MexAB and the induction of the SOS response (Brazas & Hancock, 2005), thereby decreasing intracellular levels of the drug and repairing damage to the DNA respectively. In this instance, high levels of resistance are a consequence of the transitory hypermutation state (associated with the SOS response) which facilitates the acquisition of additional resistance traits (a phenomenon referred to as adaptive mutation) (Fernández *et al.*, 2011). Sass *et al.* (2011)

demonstrated that a stable induction of multi-factorial resistance determinants can occur in *B. cenocepacia* exposed to amikacin, meropenem and trimethoprim-sulfamethoxazole antibiotics.

Adaptive resistance to biocides is not a new phenomenon, the ability of bacteria developing resistance to gradually increasing doses of chemical agents having been recognised as far back as 1887 (Russell, 2004a). Since Adair *et al.* (1969) first reported on the effect of a biocide on the adaptive resistance of *P. aeruginosa,* there have been many examples of biocide-induced adaptive resistance in several bacterial species (Fernández *et al.*, 2011). Although they are not yet fully understood, the described mechanisms leading to biocide-induced adaptive resistance are considered diverse and specific to the biocide (Fernández *et al.*, 2011). Non-specific mechanisms of resistance such as outer membrane alterations and active efflux are considered to be of particular importance in biocide adaptation (Fernández *et al.*, 2011), as generally (with the exception of triclosan), biocides possess multiple cellular target sites (Denyer, 1995; Hugo, 1967). Consequently, biocide-induced adaptive resistance is often associated with cross-resistance to antibiotics; a phenomenon which fuels concerns that the indiscriminate use of biocides in various environments contributes to the development of antibiotic resistance (Fernández *et al.*, 2011; Gilbert & McBain, 2003). Several studies have investigated resistance mechanisms leading to biocide-induced resistance and the effects on antibiotic susceptibility.

Loughlin *et al*. (2002) demonstrated that the sequential passage of *P. aeruginosa* strains in the presence of sub-inhibitory concentrations of benzalkonium chloride resulted in an increased resistance to the agent and cross-resistance to QACs (CPC and cetrimidine), polymyxin B and chloramphenicol antibiotics. Characteristics associated with resistance were alterations to the outer membrane protein profile, the uptake of benzalkonium chloride, cell surface charge and hydrophobicity, and fatty acid content of the cytoplasmic membrane (Loughlin *et al.*, 2002). Another interesting observation was that the cross-resistance profiles of adapted strains differed, indicating the mechanisms leading to benzalkonium chloride-induced adaptive resistance to be strain specific. Subsequent studies have since demonstrated this phenomenon to occur in other bacterial species. For example, Braoudaki (2004) investigated adaptive resistance to QACs, chlorhexidine and triclosan biocides in *Salmonella enterica* and *E. coli*, observing that cross-resistance to clinically relevant antibiotics varied with the serotype and the biocide.

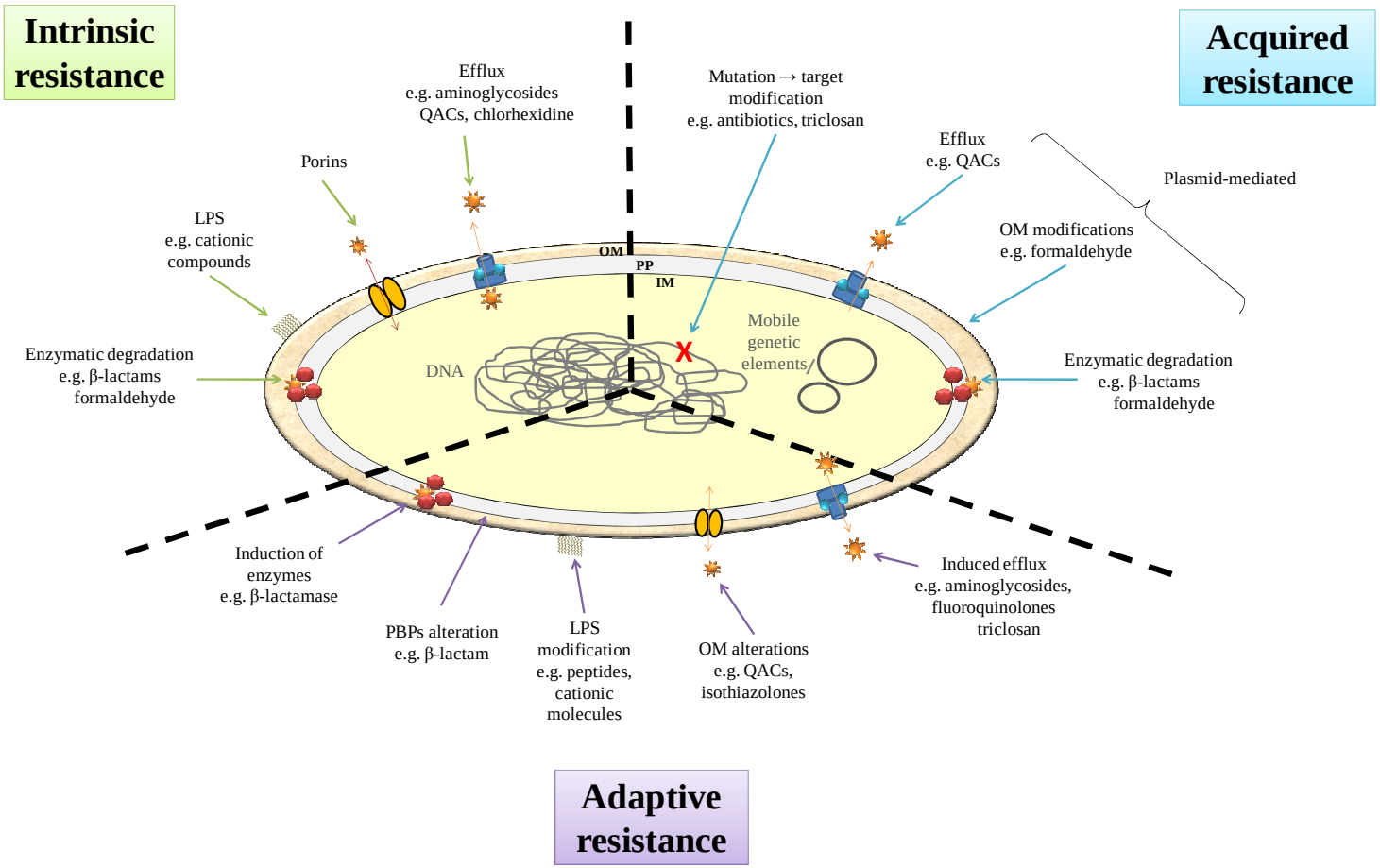


Figure 7. Intrinsic, acquired and adaptive resistance mechanisms of Gram-negative bacteria.

The schematic summarises bacterial resistance mechanisms, with examples of their involvement in antibiotic and biocide resistance, as described in section 1.4. Resistance mechanisms can be defined as: (i) an intrinsic natural property of an organism; (ii) acquired, through the mutation of chromosomally encoded genes, or via the acquisition of resistance genes encoded on mobile genetic elements such as plasmids or transposons; (iii) adaptive, that involves the induction of chromosomal, or plasmid encoded, resistance determinants in response to stimuli- such as an antimicrobial agent at sub-inhibitory concentrations.

1.5 PROJECT AIMS

This Ph.D studentship was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) in collaboration with the CASE sponsor Unilever Research UK. Since Unilever are a leading manufacturer of home and personal care products, the questions and aims investigated in this study reflect their commercial interests. The overall goal of the project was to investigate the molecular basis for preservative resistance in Bcc bacteria, the research and strategies used to achieve this are shown in Figure 8. The specific aims and hypotheses of the project were as follows:

1) **To examine the preservative susceptibility of Bcc bacteria (Chapter 3).** The minimum inhibitory and bactericidal concentrations of preservatives for a collection of genetically diverse Bcc bacteria, from various isolation sources, were determined using agar and broth dilution methods. This explored the relationship between Bcc species diversity, isolation source and preservative susceptibility. ***Hypothesis 1: the preservative susceptibility of Bcc bacteria is related to species diversity and source of isolation.***

2) **To investigate adaptive resistance to preservative agents in Bcc bacteria (Chapter 4).** The progressive subculture of *B. lata* strain 383 on increasing concentrations of preservative agents was used to explore the adaptive resistance potential of Bcc bacteria. Preservative and antibiotic susceptibility profiling of the derivatives of the parental strain with adaptive resistance was used to explore the potential for cross-resistance to other antimicrobials. ***Hypothesis 2: the stepwise exposure of B. lata strain 383 to sub-lethal preservative concentrations will promote stable adaptive resistance; in addition, this preservative-induced adaptive resistance will confer cross-resistance to other antimicrobials.***

3) **To determine the molecular basis for isothiazolinone and DMDM hydantoin preservative resistance in Bcc bacteria (Chapter 5).** Transposon mutagenesis was used to search for the genetic determinants of isothiazolinone and DMDM hydantoin preservative resistance in the genome strain *B. lata* strain 383. The aim of this work was to identify resistance mechanisms utilised by Bcc bacteria. ***Hypothesis 3: Bcc resistance to isothiazolinone and DMDM hydantoin preservatives is mediated by multiple resistance determinants.***

4) **To examine differential gene expression in response to isothiazolinone and DMDM hydantoin preservatives (Chapter 5).** A DNA microarray and transcriptomic analysis was utilised to examine global gene expression of *B. lata* strain 383 in response to sub-inhibitory concentrations of the isothiazolone blend of preservatives MIT/CMIT, and the formaldehyde donor DMDM hydantoin. The aim of this research was to identify genes and/or gene pathways involved in Bcc resistance to these preservative agents. ***Hypothesis 4: multiple preservative resistance determinants will be identified in B. lata strain 383 by the differential expression of genes and gene pathways in response to sub-inhibitory concentrations of preservative.***

5) **To determine the molecular basis for adaptive resistance preservatives (Chapter 7).** A DNA microarray and real time quantitative PCR methods was utilised to determine differential gene expression in a preservative-adapted *B. lata* strain 383 derivative. The aim of this research was to identify resistance determinants involved in Bcc adaptive resistance to preservative agents. ***Hypothesis 5: multiple resistance determinants leading to preservative-induced adaptive resistance in B. lata strain 383 will be identified by the relative differential expression of genes and gene pathways in a preservative-adapted derivative of the wild-type.***

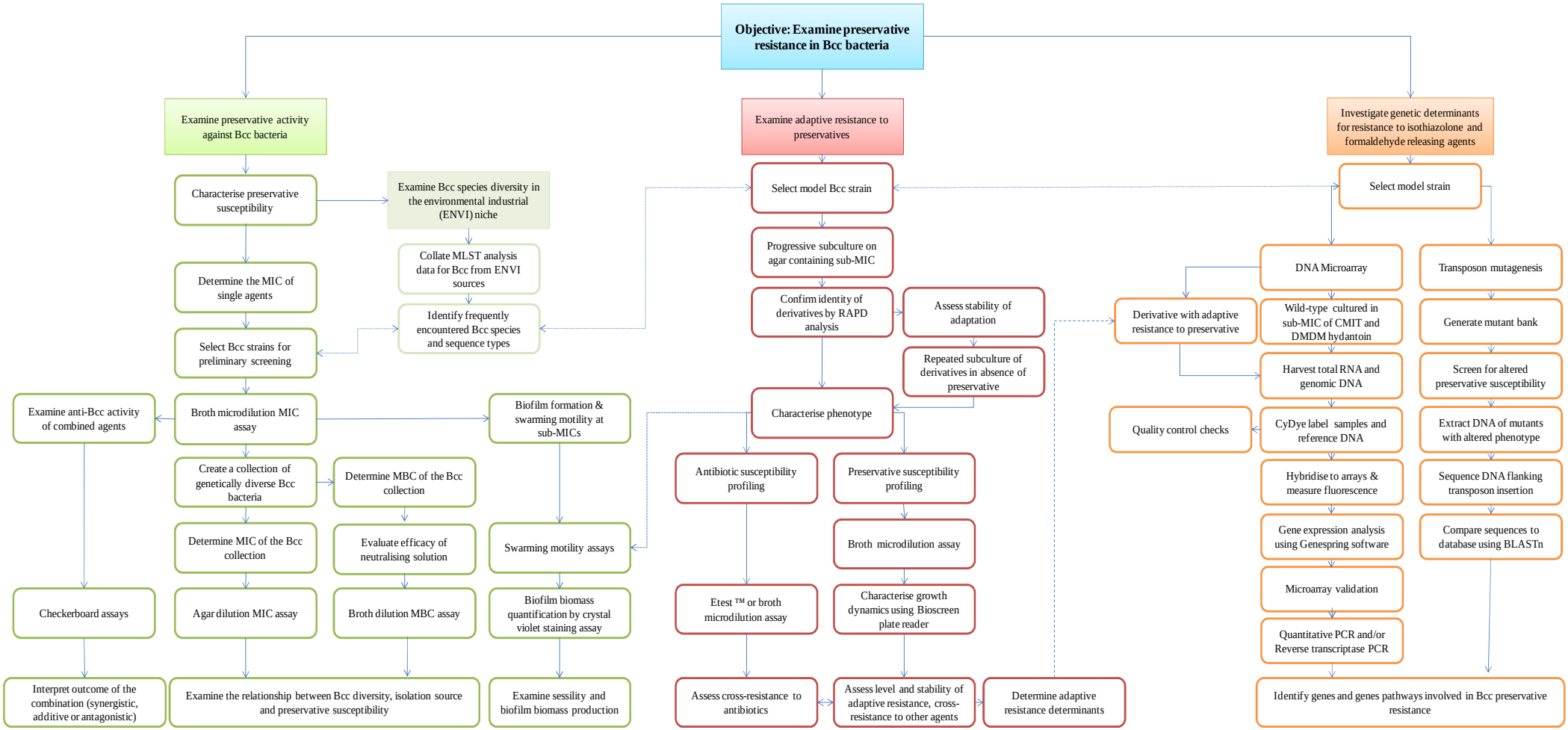


Figure 8. The main experimental approaches used to investigate preservative resistance of Bcc bacteria.

Three strategies were used to investigate Bcc preservative resistance : (i) to characterise the activity of preservatives against Bcc bacteria using cultivation-based techniques; (ii) to examine adaptive resistance to preservatives by progressive subculture in the presence of sub-lethal concentrations; (iii) to determine resistance determinants by gene expression analysis and mutagenesis . Work contained within chapter 3 is shown in green, Chapter 4 is in red and Chapters 5 & 6 are shown in orange.

2 MATERIALS & METHODS

2.1 CHEMICALS

Unless otherwise stated, the chemicals used in this study were obtained from Sigma-Aldrich (Dorset, UK), ICN Biomedicals Ltd (Oxon, UK) and Fisher Scientific (Loughborough, UK). Preservatives and preservative enhancing agents were obtained from suppliers listed in Table 2. All aqueous solutions were prepared in ultra pure deionised water ≥18MΩ cm.

2.2 PREPARATION OF ANTIMICROBIAL AGENTS

2.2.1 Preservatives and preservative enhancing agents

For the purpose of this study EDTA, Dermosoft Octiol and sensivia SC50 are described as preservative enhancing agents or potentiators. The composition of preservatives and potentiators is shown in Table 2. Stock solutions of ≤ 50% (w/v or v/v) were prepared on the day of use, and were subsequently used to prepare further preservative concentrations where required. Active ingredient concentrations (%) of the preservative agents evaluated are listed in Table 2. Stock solutions of water insoluble agents were prepared in Dimethylsulfoxide (DMSO) and were heated to 45ºC to aid the dissolving process if required.

2.2.2 Antibiotics

Antibiotic stock solutions were prepared at 100 mg/ml, except for polymyxin B (PMX) which was at 120,000 units/ml. Kanamycin (Km), ciprofloxacin (CIP), levofloxacin (LVX), norfloxacin (NOR), and sparfloxacin (SPX) antibiotics were dissolved in ultra pure deionised water; glacial acetic acid and sodium hydroxide were added to norfloxacin and to sparfloxacin respectively to aid the dissolving process. Tetracycline (Tc) and trimethoprim (Tp) antibiotics were dissolved in DMSO.

2.3 MEDIA

All media were prepared with deionised water and sterilised by autoclaving at 121ºC. A modified basal salts media (BSM) was predominantly used in this study. This consisted of: $K_2HPO_4.3H_2O$ 4.25 g/L, $NaH_2PO_4.H_2O$ 2 g/L, NH_4Cl 40 g/L, $MgSO_4.7H_2O$ 0.2 g/L,

$FeSO_4.7H_2O$ 0.012 g/L, $MnSO_4.H_2O$ 0.003 g/L, $ZnSO_4.7H_2O$ 0.003 g/L, $CoSO_4.7H_2O$ 0.001 g/L and was supplemented with 0.5g/L casamino acids (Becton & Dickinson, Sparks, USA.), 0.5g/L yeast extract, and a 0.4% (w/v) glucose carbon source, abbreviated to BSM (CYG). To avoid potentiating the activity of preservatives and preservative enhancers, nitrilotriacetic acid ($C_6H_9NO_6$ 0.1 g/L) was omitted from the media. BSM (CYG) agar was prepared with 6 g/l purified agar (Oxoid). Additional media included Tryptone soya agar/broth (TSA/TSB), Luria-Bertani agar/broth (LBA/LB), Iso-sensitest agar/broth (ISO-A/ISO-B) and Mueller-Hinton agar/broth (MHA/MHB), adjusted to contain 20-25 mg/L calcium and 10-12.5 mg/L magnesium.

2.4 BACTERIAL STRAINS

All organisms were drawn from the Mahenthiralingam group strain collection at Cardiff University. Species and strain designations for bacterial isolates are shown within Tables 5, 6, 7 and 14.

2.4.1 Growth conditions

Unless otherwise stated, bacterial isolates were revived from frozen stocks and cultured on a BSM (CYG) at 30ºC; clinical isolates often required a higher temperature of 37ºC. Overnight cultures (18 hours) were prepared by inoculating 3 ml of broth media with a single colony from a revival plate, tubes were then incubated, with shaking at 200 rpm, at 30 or 37ºC for 18 hours, depending on the growth requirements of each isolate.

2.4.2 Storage of bacterial isolates

Stocks of bacterial isolates were prepared by resuspending fresh colonial growth from a pure culture plate into BSM (CYG) broth containing 8% (v/v) DMSO. Stocks were then maintained at – 80ºC.

2.4.3 Enumeration of viable bacteria

Bacterial suspensions were enumerated using a viable drop count method. Serial dilutions were performed in BSM (CYG) broth unless otherwise stated; triplicate 10 µl drops were aspirated onto the surface of BSM (CYG) agar plates and incubated at 30 or 37ºC for 24 hours. Individual colonies were counted and the number of viable cells calculated and expressed as colony forming units per ml (CFU/ml).

2.4.4 Bacterial growth in modified basal salts media

The effect of modifying basal salts media (e.g. omitting the chelating agent nitrilotriacetic acid) on bacterial growth was investigated using nine Bcc strains: *B. arboris* (Bcc1306), *B. cenocepacia* J2315 , *B. cenocepacia* (HI2424), *B. cenocepacia* (Bcc1283), *B. cenocepacia* (Bcc1291), *B. contaminans* (LMG 23255), *B. lata* strain 383 (LMG 22485), *B. lata* (Bcc1294) and *B. lata* (Bcc1296) . Strains were revived and cultured for 18 hours in BSM (CYG) broth containing nitrilotriacetic acid as described in section 2.4.1, and diluted to an optical density (OD) of 1±0.2 (600 nm). Flasks containing 25 ml of BSM (CYG) broth with and without nitrilotriacetic acid were inoculated with 1 x 10^5 CFU and incubated at 30°C on an orbital shaker at 150 rpm. Viable cells were enumerated at 0, 8 and 24 hours, as described in section 2.4.3; using BSM (CYG) agar with or without nitrilotriacetic acid for serial dilutions and agar plates as appropriate. Significant differences ($P<0.05$) in the number of viable cells were determined using a Mann-Whitney (two-tailed) statistic test, performed using the statistical software Minitab V.16.

2.5 DNA EXTRACTION FROM BACTERIAL CELLS

2.5.1 Rapid DNA extraction from colony material

A single bacterial colony from a pure culture plate (with $\leq$ 72 hours' growth) was aseptically transferred to 50 µl of 5% (w/v) chelex® 100 resin solution (Biorad, Hertfordshire, UK, sterilised by autoclaving prior to use). DNA extraction was performed by heating the sample to 98°C on a heated block for a 5 minute cycle, then immediately placing the sample at 4ºC for 5 minutes. This process was repeated twice. Samples were then centrifuged briefly at 800 x *g* to sediment the resin and cellular debris and the supernatant containing the crude DNA removed for subsequent use.

2.5.2 Genomic DNA extraction: Bead-beater method

Overnight (18 hours) cultures of the bacteria were set up and harvested by centrifugation (1,400 x *g* for 10 minutes). The pellet was resuspended in 100 µl of TE buffer (10 mM Tris-Cl pH8, 10 mM EDTA pH8) and transferred to a microtube containing approximately 500 µl of 0.1 mm diameter washed zirconium beads (Biospec Products, Bartlesville, Oklahoma) and 500 µl lysis buffer (50 mM Tris-CL pH8, 50 mM EDTA pH8, 1% (w/v)

SDS) with 0.5 mg/ml Pronase (Boehringer/ Roche). Bacteria were lysed by pulsing a microtube on a mini bead-beater machine (Biospec Products) for 10 seconds, and proteins were digested during incubation for 1 hour at 37°C. 200 µl of saturated ammonium acetate to lysate was added and mixed by a 5 second pulse on the bead-beater machine. Samples were briefly centrifuged at 15,000 x *g*, followed by the addition of 600 µl chloroform. Samples were pulsed on a bead-beater device for 5 seconds and then centrifuged for a further 7 minutes at 15,000 x *g*, to separate the phases. DNA was extracted from the clear upper aqueous phase by ethanol precipitation. The DNA pellet was washed with 70% (v/v) ethanol, dried under vacuum and re-suspended in low-EDTA TE buffer (10mM Tris-Cl pH8, 0.1mM EDTA) with RNase A at 0.5 µg/ml. High molecular weight genomic DNA was quantified using a Nanodrop 1000 (Thermofisher Scientific, Leistershire, UK) and stored at -20ºC for subsequent use.

2.6 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

RAPD analysis was carried out as described by Mahenthiralingam *et al.* (1996). Primer 272, 5'- TGC GCG CGG G -3' (MWG Biotech, Covent Garden, London) was used for all reactions; reagents were from Qiagen (Qiagen, Crawley, Sussex, UK). Profile analysis was performed in 25 µl reaction mixtures containing: 1x PCR buffer, 1 x Q-solution, 3 mM $MgCl_2$, 200 µm dNTPs mixture, 1.6 µM RAPD primer, 1 U of *Taq* polymerase and 2 µl of DNA template (either used neat if obtained by Chelex protocol or diluted 1 in 10 if high molecular weight genomic DNA). PCR thermal cycles were performed using a Flexigene Thermal Cycler (Techne Ltd., Newcastle, United Kingdom) as follows: 5 minutes at 94ºC, 4 cycles of 5 minutes at 36ºC, 5 minutes at 72ºC, 5 minutes at 94ºC, 30 cycles of 1 minute at 94ºC, 1 minute at 36ºC, 2 minutes at 72ºC followed by a final extension time of 10 minutes at 72ºC. The PCR product was visualised on a 1.5% (w/v) high resolution agarose gel as described by Mahenthiralingam *et al.* (1996) or 1 µl of the product was run on an Agilent Bioanalyser 2100 (Agilent) using a DNA 7500 chip according to the manufacturer's protocol.

2.7 RNA EXTRACTION FROM BACTERIAL CELLS

2.7.1 Harvesting bacterial cells

Flasks containing 25 ml of BSM (CYG) broth were inoculated with 2 x 10^8 CFU of a bacterium and incubated at 30°C on an orbital shaker at 150 rpm. Experimental cultures for microarray analysis (Section 2.9) were supplemented with 0.00162% (v/v) dimethylol dimethyl hydantoin or 0.00001498% (v/v) of the blend of methylisothiazolinone and chloromethylisothiazolinone preservatives. Growth was monitored spectrophotometrically throughout the incubation. Cultures harvested at mid-exponential growth phase (OD of 0.5 at 600 nm, 2 x 10^8 CFU/ml) were promptly aliquoted into a microcentrifuge tube and immediately snap-cooled in liquid nitrogen before centrifuging at 20,000 x *g* at 4ºC for 1 minute. The supernatant was removed and pellets were immediately frozen at -80ºC. Experiments were repeated with different starting cultures to obtain three biological replicates.

2.7.2 Total RNA extraction

Total RNA was extracted within one week of harvesting the cultures using the RiboPure™ Bacteria Kit (Ambion, Applied Biosystems, Texas, US) according to the manufacturer's instructions. This method combines the glass bead-mediated mechanical disruption of cells with a phenol based detergent and denaturant (RNAwiz), followed by a glass filter-based RNA purification. In brief, two cell pellets are resuspended in 350 µl of RNAwiz. Cells were disrupted using Zirconia beads by a 10 second pulse on a bead-beater device. 0.2 volumes of chloroform were added to the lysate and the aqueous and organic phases separated by centrifugation. The upper aqueous phase containing the RNA was removed, washed with ethanol and purified using a silica filter. Each sample was treated with DNase I (Ambion) for 60 minutes to remove any contaminating genomic DNA.

2.7.3 Lithium chloride purification and concentration of RNA

RNA samples were adjusted to 200 µl and 100 µl of ice cold 7.5 M Lithium chloride (LiCl) (Ambion) added. Samples were placed at -20ºC for 30 minutes. RNA was pelleted by centrifugation at 16,000 x *g* at 4ºC for 30 minutes, and the supernatant removed. Pellets were washed in 70% (v/v) ethanol, centrifuged at 16,000 x *g* at 4ºC for 15 minutes, and air-dried under vacuum for 30 minutes. Each pellet was resuspended in 15 µl of RNase-

free water and the concentration of RNA determined using the Nanodrop 1000 (Thermofisher scientific). Aliquots were adjusted to an RNA concentration of 150 ng/µl and subjected to a second DNase treatment; 2 µl of DNase I enzyme (Promega, Hampshire, UK) was added to 7 µl RNA and 10 X buffer (Promega). After a 1 hour incubation at 37ºC the reaction was terminated by adding 1 µl of stopping reagent and heating samples to 65ºC for 10 minutes. The RNA quality was assessed with a Bioanalyzer (Agilent Technologies Ltd, Berkshire, UK) using the RNA 6000 Nano kit (Agilent). This RNA was then used to synthesize cDNA for use in reverse transcriptase PCR, real-time quantitative PCR and microarray experiments.

2.8 GENE EXPRESSION METHODS

2.8.1 Generating cDNA for PCR-based methods

RNA was converted to cDNA using an Improm-II Reverse Transcription System (Promega). This system was also designed to generate several controls including, a negative control without RNA (no-RNA) to control for template contamination and an internal positive control (template and control primers included in the kit) to check activity of the reverse transcriptase enzyme. In addition, experimental reactions without reverse transcriptase (no-RT) were included to control for genomic DNA or plasmid contamination. 3 µl of RNA template and 1.25 µl of random primers (Fisher Scientific) were made up to 5 µl with nuclease-free water for experimental samples. Negative control samples did not contain RNA template. RNA template and random primers reaction mixes were first denatured by heating to 70ºC for 5 minutes and then chilled at 4ºC for 5 minutes. RNA was then converted to cDNA in the following reaction mixture: 4 µl of buffer, 4.8 µl of 25 mM $MgCl_2$ 1 µl of 10 mM dNTP, 0.5 µl of RNasin inhibitor and 1 µl of Improm reverse transcriptase (or nuclease-free water for no-RT controls), and made up to 15 µl with nuclease-free water. 5 µl of the denatured primer/RNA mix was added to the reaction mixture (except the no-RNA control) and placed in a thermal cycling block. The following cycles were used to generate cDNA : annealing for 5 minutes at 25ºC, extension for 60 minutes at 42ºC and 15 minutes at 70ºC to deactivate the reverse transcriptase step. cDNA was then stored at – 20ºC for subsequent use.

2.8.2 Reverse transcriptase PCR (rtPCR)

Reverse transcriptase PCR, a semi-quantitative method, was used in conjunction with quantitative real-time PCR (qRT-PCR) to validate the DNA microarray. rtPCR was used to investigate the differential expression of genes B0668 and A3949 (under control conditions, no preservative) in the adapted derivative *B. lata* 383-CMIT relative to expression levels in the parental strain. Real-time PCR of these genes was not possible due to limited sensitivity. A housekeeping gene encoding acetoacetyl-CoA reductase (*phaC*) was included as a control as it had constant levels of expression across all conditions. A second housekeeping gene, recombinase A (*recA*), was also included as a control as it had constant levels of expression under control conditions. Primers complimentary to the four genes were designed (Table 4) cDNA was generated as previously described (section 2.8.1) and 2 µl was added to the following Qiagen reagents: 5 µl PCR buffer (10x), 10µl q-solution (5x), 1 µl dNTPs (10 mM), 4 µl of 1:1 primer mix (10 µm), 0.25 µl *Taq* (5 units/µl), and made up to 25 µl with nuclease-free water. Reaction conditions for all primers were as follows: initial denaturation at 96ºC for 1 minute; 30 cycles of denaturation at 96ºC, primer annealing at 55ºC for 1 minute, extension at 72ºC for 1 minute followed by a final extension step of 72ºC for 10 minutes. PCR products were analysed for differential amplification (and therefore differential expression) after 20 and 30 cycles by visualisation on a 2% (w/v) agarose gel.

2.8.3 Quantitative PCR (qRT- PCR)

In contrast to rt-PCR which is only semi-quantitative, real-time PCR is highly sensitive and allows accurate quantification of transcripts and small changes in gene expression (Heid *et al.*, 1996; Pfaffl, 2001). qRT-PCR was used to validate microarray results and to investigate expression levels of the gene B1004 in *B. lata* strains. The general qRT-PCR approach used in this study has been previously described by Drevinek *et al.* (2008). qRT-PCR was performed in triplicate using an ABsolute QPCR SYBR® Green kit (ABgene, Epsom, UK) with $MgCl_2$ concentration of 3 mM.. Primers were designed to target five genes with altered gene expression in the adapted derivative *B. lata* 383-CMIT and two control genes of constant expression (Table 4). Target genes were selected to represent the range of expression changes observed in the microarray experiments (from 1.9 to 25 fold). cDNA was generated (as previously described section 2.8.1) and 2 µl was added to 10 µl of ABsolute SYBR green mix, 0.4 µl of forward and reverse primers (20 µM) and made up to 20 µl with nuclease-free water. A standard curve was generated using 1 in 5, 1 in 25 and

1 in 50 dilutions of the cDNA template, to assess the efficiency of amplification. Internal controls of 'no-RT' and 'no-cDNA' were included to control for genomic DNA contamination and primer-dimer artefacts respectively. Expression levels of housekeeping genes *phaC* and/or *recA* were used to normalise data. Amplifications were run on a MJ Research PTC-200 thermal cycler with the option of real-time fluorescence detection (DNA Engine Opticon; Bio-Rad Laboratories, Hertfordshire, UK). Cycling conditions were as follows: after an initial 15 minute thermal activation of the modified Taq-polymerase at 95ºC, 50 cycles of 15 seconds at 95ºC, 30 seconds at 62ºC and 30 seconds at 72ºC were performed. Data were obtained at 72ºC and melting curve analysis was performed at the end of the PCR to test for specific PCR product. Fold change was calculated using the threshold cycle (C_T) of each reaction i.e. the PCR cycle number at which the accumulating product yields a detectable fluorescent signal. Two methodologies were applied to quantify the relative expression levels of the target genes (i.e the PCR signal of the target transcript in adapted *B. lata* derivatives or other *B. lata* strains relative to the calibrator strain *B. lata* 383).

2.8.3.1 Analysing qRT-PCR data using the comparative CT method

The comparative C_T or delta-delta (also known as the $2^{-\Delta\Delta\ CT}$) (Livak & Schmittgen, 2001) assumes both target and reference genes are amplified with efficiencies near 100% and within 5% of each other (Schmittgen & Livak, 2008). The relative difference in expression was calculated by first normalising the C_T of the target gene to the reference gene (*phaC* or *recA*) for both the test and the calibrator samples. The normalised C_T values of the sample of interest are then compared to the normalised C_T values of the calibrator (*B. lata* strain 383).The equation used was as follows:

[delta][delta] C_T= [delta] $C_{T\ sample}$ - [delta] $C_{T\ calibrator}$

Here, [delta] $C_{T\ sample}$ denotes the C_T value for the test condition (e.g. *B. lata* 383-CMIT) normalised to the housekeeping gene *phaC* or *recA* and [delta] $C_{T\ calibrator}$ is the C_T value for the control condition or "calibrator" (*B. lata* strain 383).

2.8.3.2 Analysing qRT-PCR data using the Pfaffl method

The Pfaffl method accurately calculates the relative difference in expression as it uses calculated PCR efficiencies and normalised C_T values of the sample of interest versus the calibrator (Pfaffl, 2001). Standard curves were generated, by serial dilution of cDNA from test samples and the calibrator *B. lata* 383, for target and reference genes. The amplification efficiencies of each target and reference gene were calculated from the slope of the standard curve using the following formula:

$$\text{Efficiency} = 10^{-1/\text{slope}}$$

The expression ratio between test samples and the calibrator was then calculated using the following formula:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT\ \text{Target (calibrator} - \text{test)}}}{(E_{\text{reference}})^{\Delta CT\ \text{Reference (calibrator} - \text{test)}}}$$

Here, E_{target} is the amplification efficiency of the target gene transcript; $E_{\text{reference}}$ is the amplification efficiency of the reference gene transcript.

Table 4. Primers used in reverse transcriptase PCR and quantitative PCR

Primer name	Nucleotide Sequence (5` – 3`)	Amplicon size (bp)
phaC_RT fw	AAGCGTTCGACAAGGTCAAG	258
phaC_RT rv	CGGTCGAGTAGTTGGTCTGG	
recA_RT fw	AGAACATCCAGGTCGTGTCC	214
recA_RT rv	TTCGCTGCATATTGAACGTC	
B1004_RT fw	CCCCGTCTACGTGTATTTCG	230
B1004_RT rv	TAAACGTATGGTCCGCATTC	
B1327_RT fw	CATGTCGGTGTTTCCGTTC	279
B1327_RT rv	ATGAACGTGCTCCACAGTCC	
B1767_RT fw	ACCTGATCAAGCTGCTCACC	275
B1767_RT rv	CTTGTCCTTCTTTGCCTTCG	
A6485_RT fw	TTTCTGGGCTTTCGATGATG	280
A6485_RT rv	CGCATGAAGTTCGTGTTCAG	
B0668_RT fw	CTGGACGCCGACATCATC	228
B0668_RT rv	CCGCCAATACTGCGTCTG	
A3516_RT fw	TGTTCCTGATCCCCGTATTC	288
A3516_RT rv	GCTTGAACTGCTCCTGAACC	
A3949_RT fw	CGTACTTCCCGCTCGTCTAC	239
A3949_RT rv	CGCATAGATCTCGGTCGTG	

Footnotes:

fw, forward primer; rv, reverse primer.
bp, base pair.

2.9 MICROARRAY EXPERIMENTS

A two-colour microarray of *B. lata* strain 383 was used to determine global gene expression in response to a cosmetics grade blend of methylisothiazolinone and chloromethylisothiazolinone preservatives, and a DMDM hydantoin preservative. In addition, global gene expression of the preservative adapted derivative *B. lata* 383-CMIT, in the absence and presence of isothiazolinone preservatives, was also explored.

A custom 4x44k microarray for *B. lata* strain 383 was designed by Oxford Gene Technology (Oxford Gene Technology, UK) and manufactured by Agilent (Agilent technologies, Santa Clara, California) (design #025314) using high-density 60-mer SurePrint technology. The microarray was composed of 14132 probes: 14071 probes were derived from coding genes and intergenic regions in the *B. lata* strain 383 genome, and 61 probes served as internal controls. Each probe was printed three times, with a randomised distribution. Experimental protocols and raw data can be found in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MEXP-2827 (withheld until 20/07/2011).

2.9.1 Growth curve analysis

To ensure consistent RNA profiling, cells were harvested at mid-logarithmic growth phase. Growth curve experiments were performed to determine suitable sub-inhibitory concentrations of preservatives for the microarray experiments. The minimum inhibitory concentrations (MIC) of the isothiazolinone blend and DMDM hydantoin preservatives were determined for *B. lata* 383 and *B. lata* 383-CMIT using a broth dilution method (see section 2.9.1). Appropriate amounts of aqueous stock solutions of preservatives were added to BSM (CYG) broth to achieve test concentrations approximately one quarter or half that of the wild-type MIC: 0.00001% (v/v), 0.00001498% (v/v) for the isothiazolinone blend; 0.00162% (v/v), 0.0027% (v/v) for DMDM hydantoin. Briefly, flasks with 25 ml of BSM (CYG) broth, with and without preservative, were inoculated with 2 x 10^8 CFU and incubated at 30° C on an orbital shaker at 150 rpm. Growth was monitored spectrophotometrically over 9 hours. Sub-inhibitory concentrations for microarray experiments were selected based on the following criteria: (1) exposure to the preservative resulted in altered growth dynamics but did not prevent cells entering a logarithmic growth phase; (2) an optical density of 0.5 at 600 nm was reached within 8 hours. The method for

harvesting cells and subsequent extraction of total RNA is described in sections 2.7.1. and 2.7.2.

2.9.2 Labelling first strand cDNA with amino allyl-dUTP

A Superscript™ Indirect cDNA Labelling System (Invitrogen, UK) was used to synthesise first-strand cDNA incorporating chemically reactive nucleotide analogues (amino allyl-dUTP). 10 µg of total RNA were used per labelling reaction containing 2.2 µl random hexamers (400 ng/µl), and made up to 18 µl with DEPC- treated water. Tubes were incubated at 70ºC for 5 minutes and placed on ice so primers could anneal. The following reagents were added for the extension reaction: 6 µl 5 x first strand buffer; 1.5 µl 0.1 M DTT; 1.5 µl dNTP mix (including amino-modified nucleotides); 1 µl RNaseOUT™; 2 µl SuperScript™ III reverse transcriptase. Reactions were incubated at 46ºC for 3 hours.

2.9.3 Degradation of RNA and purification of amino allyl modified cDNA

After cDNA synthesis a hydrolysis reaction was performed to degrade the original RNA. 15 µl of 1 N NaOH was added to each reaction after gentle mixing tubes were incubated at 70ºC for 10 minutes. 15 µl of 1 N HCL was added and gently mixed to neutralise the pH, followed by 20 µl of 3 M Sodium acetate (pH 5.2). Unincorporated dNTPs and hydrolysed RNA were removed using an Illustra CyScribe GFX purification kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's protocol. cDNA was eluted in 2 x 50 µl of DEPC-treated water (heated to 65ºC), and purified by ethanol precipitation. 10 µl of 3 M sodium acetate (pH 5.2) was added to each tube followed by 2 µl of 20 mg/ml glycogen, and mixed. 300 µl of ice-cold ethanol was added, and tubes incubated at -20ºC for 1 hour. The cDNA was pelleted by centrifugation at 14,000 xg at 4ºC, and the pellet washed in 80% (v/v) ethanol. After centrifugation at 14,000 xg for 2 minutes, the supernatant was discarded and the pellet air-dried. cDNA was resuspended in 5 µl of 2X coupling buffer (Invitrogen).

2.9.4 Labelling of amino-allyl modified cDNA with Cy5™ Dye

The modified first strand of cDNA was chemically labelled with Cy5™ dye (Amersham Biosciences Ltd, Buckinghamshire, UK) in a separate step, to minimise any dye bias. CyDye was resuspended to individual reaction size, by adding 5 µl DMSO, and added to the purified amino-allyl modified cDNA /coupling buffer suspension. Reactions were gently mixed and incubated at room temperature (20 °C) in the dark for 1 hour.

2.9.5 Purification of Cy5-labelled cDNA

An Illustra Cyscribe GFX purification kit (GE Healthcare) was used to purify the labelled cDNA, removing unreacted dye. The kit was used according to manufacturer's instructions with one exception. To maximise recovery, purified Cy5-labelled cDNA was eluted from columns with 60 µl DEPC-treated water (heated to 65º C) and incubated for 5 minutes at room temperature before collection by centrifugation. Samples were stored in protective amber microtubes at -20ºC for subsequent analysis.

2.9.6 Labelling reference genomic DNA with Cy3™ Dye

A DNA reference design was used for the microarray experiments with Cy3-labelled genomic DNA, from *B. lata* strain 383, run as the control channel for all experiments. Genomic DNA (extracted as described in section 2.5.2) was directly labelled with Cy3 Dye (Amersham) using a BioPrime® DNA labelling system (Invitrogen). 2 µg of genomic DNA was added to a sterile microtube and the volume made up to 21µl with DEPC-treated water. 20 µl of a 2.5x random primer/reaction buffer mix (supplied in the kit) was added. Samples were placed on a thermal block and heated to 98ºC for 5 min, and then placed on ice for 5 minutes. While on ice, the following reagents were added to the reaction mix: 5 µl of a 10x dNTP mix (1.2 mM each dATP,sGTP,dTTP; 0.6 mM dCTP; 10mM Tris pH8 ; 1 mM EDTA), 3 µl of Cy3 dCTP (1 mM stock) and 1 µl of klenow enzyme (supplied in kit). Reaction mixtures were incubated in the dark at 37ºC for 2 h. The reaction was terminated by the addition of 5 µl of stop buffer (supplied in the kit).

2.9.7 Purification of Cy3-labelled reference DNA

An Illustra GFX PCR DNA and Gel Band Purification kit was used to purify Cy3-labelled reference DNA, removing unincorporated/quenched Cy3 Dye. The kit was used according to the manufacturer's, instructions with one exception: to maximise recovery, purified Cy3-labelled DNA was eluted from columns with 2x 30 µl DEPC-treated water (heated to 50ºC) and incubated for 5 minutes at room temperature before collection by centrifugation. Samples were stored in protective amber microtubes at -20ºC for subsequent analysis.

2.9.8 Quality control of CyDye labelled nucleic acids

A Nanodrop 1000 spectrophotometer (Thermo Fisher) was used to quantify the concentration of generated cDNA, reference DNA and the amount of CyDye incorporated.

Optimal concentrations of cDNA and CyDye ranged from 20 to 70 ng/µl and 2.5 to 5.0 pmol/µl respectively. Higher dye levels are an indication of unincorporated dye and may increased the background noise on the array. In addition, fluorescence was measured by electrophoresis on a miniature agarose gel (3 ml of 1.5% w/v agarose gel containing 1 x TAE (40 mM Tris acetate, 1 mM EDTA) poured into the gel mould and overlaid with a glass microscope slide). 1 µl of the sample was mixed with 1 µl 50% glycerol and loaded into the gel and electrophoresed at 120 V for 20 min. The gel, mounted on the glass slide, was scanned at 560 nm and 675 nm (Gene TAC LS IV, Genomic solutions) to visualise the fluorescence of Cy3-labelled reference DNA and Cy5-labelled cDNA respectively.

2.9.9 Hybridisation

Microarrays were hybridised according to the Two-Colour Microarray Based Gene Expression Analysis protocol version 5.5, (February 2007, order number G4140-90050, Agilent) adjusted for the use of cDNA by omitting the fragmentation step. Labelled cDNA was used at 825 ng per sample, labelled reference DNA at 60 ng. Hybridisation samples were prepared as follows: 1000 ng of Cy5-labelled cDNA, 70 ng of Cy3-labelled reference DNA, 11 µl 10X blocking agent and made up to 55 µl with nuclease-free water. Samples were heat denatured at 98ºC for 3 minutes then allowed to cool to room temperature. 55 µl of 2X GEX Hybridisation buffer was then added to the denatured hybridisation mixture. Samples were mixed, avoiding the introduction of bubbles, and briefly centrifuged. Agilent microarray hybridisation chambers were assembled and loaded with clean gasket slides as instructed by the manufacturer. 102 µl of the hybridisation mixture was slowly dispensed into the gasket wells, empty wells were loaded with 102 µl of 1X hybridisation buffer (Agilent).The Agilent array was placed active side down onto the gasket slide and the hybridisation chamber clamped. The assembled chamber was rotated vertically, to wet the gasket wells and assess the mobility of bubbles within the hybridising mixture, then placed in a rotisserie in a hybridisation oven at 65ºC for 17 hours.

2.9.10 Washing slides

The washing procedure included the use of Agilent Stabilisation and Drying solutions, designed to protect against ozone-induced degradation of cyanine dyes. The wash procedure was conducted in five slide-staining dishes, containing Agilent wash buffer or solution and a magnetic stirring bar, placed on magnetic stirring plates. In brief, after

hybridisation the array-gasket sandwiched slides were removed from the chamber, submerged in Gene Expression Wash Buffer 1 (without magnetic stirrer) and carefully separated. The array slide was placed in a slide rack and submerged in fresh Wash buffer 1 at room temperature for 1 minute. The slide rack was then transferred and submerged in Gene Expression Wash Buffer 2 at 37ºC for 1 minute, followed by 1 minute in an acetonitrile wash at room temperature. Finally the slide rack was submerged in the Stabilisation and Drying solution for 30 seconds at room temperature. The slide rack was removed slowly from the final wash to avoid precipitates forming on the array slides.

2.9.11 Scanning the microarray

Microarray slides were scanned, at a resolution of 5 µm, using a microarray scanner (G2565 BA, Agilent) with Scan Control software version A.7.0.3 February 2007 (Agilent). The Extended Dynamic Range function with 100% and 10% PMT gain was enabled. Scanned images were analysed and data extracted using Feature Extraction version 9.5.1 software (Agilent), using the FE protocol GE2_v5_95.

2.9.12 Microarray data analysis

After scanning, data were imported into GeneSpring GX version 7.3.1. (Agilent) and preprocessed with the Agilent FE data import plug-in. Normalisation was performed with the Agilent FE saved scenario as follows. Firstly, every spot of the signal channel was divided by the control channel. Each chip was then normalised to the 50th percentile of all measurements of that chip, then each gene was normalised to its median. Analysis was performed using a list of 7749 coding sequences (CDS) and 6324 intergenic (IG) regions of the *B. lata* genome. Changes in gene expression in the *B. lata* derivative 383CMIT were defined by fold change relative to expression levels in the parental strain. Unreliable features were removed from analysis; a P value of ≤ 0.05 was required in at least half the samples for a feature to be included in subsequent analysis. Analysis was then performed with a 1.5 fold change filter applied. One-way analysis of variance (ANOVA) was performed on the resulting gene lists using a Welch t-test with 5% false discovery rate and no multiple testing correction. Raw data can be found in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under the accession number E-MEXP-2827 (withheld until 20/07/2011); gene lists are provided on CD-Rom in appendices.

2.10 TRANSPOSON MUTAGENESIS EXPERIMENTS

A mini-transposon Tn5 (pUT-Tn5) (de Lorenzo & Timmis, 1994) conferring Kanamycin or tetracycline resistance and the plasposon pTn*Mod*-OTp' (Dennis & Zylstra, 1998) conferring resistance to trimethoprim were considered for use in the mutagenesis of *B. lata* strain 383 (LMG 22485). Preliminary experiments were performed to determine a suitable antibiotic selective marker. An agar dilution method revealed that *B. lata* strain 383 was intrinsically resistant to kanamycin and tetracycline antibiotics. Therefore, B. *lata* strain 383 was subjected to transposon mutagenesis using plasposon pTn*Mod*-OTp', a Tn5 derivative carrying a trimethoprim resistance cassette (Dennis & Zylstra, 1998).

2.10.1 Generation of plasposon mutant bank in *B. lata* strain 383

2.10.1.1 Growth conditions

B. lata strain 383 was cultured at 30ºC on TSA/TSB; mutants were cultured at 30ºC on TSA/TSB containing 100 µg/ml trimethoprim (TSA/B-Tp100) and/or 120 units/ml polymixin were required (TSA/B-Pmx). *Escherichia coli* HB101, carrying the self transmissible "helper" plasmid PRK2013 with a kanamycin resistance cassette, was cultured at 37ºC on LB agar/broth containing 50 µg/ml kanamycin (Km50). *E. coli* JM109, carrying plasposon pTn*Mod*-OTp' with a trimethoprim resistance cassette, was cultured at 37ºC on LB agar/broth containing 100 µg/ml trimethoprim (Tp100). Transconjugants, carrying the plasposon, were selected on TSA containing 120 units/ml polymixin (Pmx120, added to counter-select against the *E. coli* donor strain) and 100 µg/ml trimethoprim.

2.10.1.2 Mating protocol

The plasposon was introduced to the *Burkholderia* strains via conjugal transfer during a triparental mating as described by Dennis and Zylstra (1998). In brief, 3 mls of 18 hour cultures of *E. coli* donor strains and a 3 mls of an 18 hour culture of the recipient *B. lata* strain 383 were harvested by centrifugation at 1,400 x *g* for 10 minutes. The retained *B. lata* and *E .coli* cell pellets were re-suspended in 3 ml or 500 µl of LB broth plus 10 mM $MgSO_4$ respectively. 100 µl of each culture was plated onto transconjugant selective media as a control for background growth in the presence of the antibiotic. Donor and recipient

cell suspensions were equally mixed (1:1:1), and 100 µl incubated at 37°C for 24 hours on a sterile 25 mm (2 µm pore size) nitrocellulose filter disc placed on the surface of a warm dry non-selective LB agar plate containing 10 mM $MgSO_4$. Filters were vortexed in l ml LB broth and 100 µl of the mating mix was plated onto transconjugant selective media and incubated at 30°C for 24 hours.

2.10.1.3 Generation of the mutant bank

Individual transconjugant colonies were randomly picked using a sterile toothpick into 185 µl TSB-Tp20 in sterile 96-well microtitre plates and incubated at 30°C, 150 rpm shaking, for 18 hours. 16 µl of DMSO was added to each well (~8% final concentration) as a cryoprotectant and the master plates stored at – 80°C for future use. After screening for preservative susceptibility, mutants of interest were streaked to single colonies from the master plate onto TSA-Tp20 from which overnight cultures were inoculated for DNA extraction.

2.10.2 Screening of mutants for putative auxotrophy

Using a multi-point inoculator (Boekel Inc.,Feasterville.PA) 120 mm square TSA and BSM agar (4 g/L glucose; not supplemented with casamino acids or yeast extract) plates were inoculated from each master plate and incubated at 30 °C for 24 hours. Putative auxotrophy was defined as 'no growth', or 'a considerable reduction in growth (e.g. microcolonies)' on the minimal BSM agar from that observed on the complex TSA plate.

2.10.3 Screening of mutants for altered preservative susceptibility

An agar dilution assay was used to identify mutants with increased or decreased susceptibility to methylisothiazolinone, DMDM hydantoin, and the blend of isothiazolinone preservatives. After autoclaving, BSM (CYG) agar was cooled to 50ºC and an appropriate volume of preservative stock solution was added to achieve final concentrations equivalent to the MIC for *B. lata* strain 383 (Wild-type), half the MIC and four-fold lower than the MIC: the MICs were 0.00097% v/v, 0.0135% v/v and 0.0000749% v/v for methylisothiazolinone, DMDM hydantoin and the blend of isothiazolinone preservatives respectively. Control BSM (CYG) agar plates without preservative and BSM (CYG) agar plates containing 100 µg/ml trimethoprim (BSM (CYG) -Tp100) were also prepared. Using a multi-point inoculator, a revival plate with

180 µl BSM (CYG) broth per well was inoculated from each master plate and incubated at 30ºC, with shaking at 150 rpm, for 18 hours. The revived mutants were then replica plated onto triplicate BSM (CYG) agar control plates, BSM (CYG) -Tp100 and BSM (CYG) test plates containing preservative and incubated at 30ºC for 72 hours. To confirm a phenotypic change, mutants of interest with a putative altered susceptibility profile were rescreened on BSM (CYG) test plates as described in this section.

2.10.4 Sequencing of transposon flanking DNA and bioinformatic analysis

Genomic DNA was extracted from mutants with an increased susceptibility and/or tolerance to the preservatives evaluated using the protocol described in section 2.5.2. RAPD profiling analysis was used to confirm the identity of the *B. lata* strain-383 mutants (section 2.6). A nested PCR approach was used to amplify genomic DNA flanking the transposon insertion site (Manoil, 2000; O'Sullivan *et al.*, 2007). Primer 1 and Primer 3 were designed to be used in conjunction with Primer 2b and Primer 4 as described by O'Sullivan *et al.* (2007). All primers were supplied by MWG Biotechnology, UK. To validate Primer 1 (5'-AGG CTC AGT GCA AAT TTA TCC-3') and Primer 3 (5'- TTG AAC GTG TGG CCT AAG CGA GC-3') each was used separately in conjunction with Primer Pc (5'-CGT CAC CAT TTG GGA GCA CAT GC-3'); a reverse primer targeting the RP4 origin of transfer in the transposon. Validation of the paired primers was carried out in a Peltier thermocycler (DYAD™) using the following cycling conditions: 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. Each 25 µl reaction contained: 1x PCR buffer, 1 x Q-solution, 3 mM $MgCl_2$, 200 µm dNTPs mixture, 1 µM of each primer, 1 U of *Taq* polymerase and 2 µl of a 1:10 dilution of genomic DNA as template. PCR products were visualised by electrophoresis on a 1.5% agarose gel. The expected amplicon size of the Primer Pc paired with Primer 1 and Primer 3 was calculated as 458 bp and 426 bp respectively.

The first round of nested PCR was carried out with Primer 1, and Primer 2b (5'-GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN ACG CC-3') in a Peltier thermocycler (DYAD™) according to the following cycling conditions: 95°C for 5 minutes, 8 cycles of 94°C for 30 seconds, 30°C for 35 seconds and 72°C for 45 seconds, a single step of 95°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 30°C for 35 seconds and 72°C for 45 seconds. Each PCR reaction contained 1U of *Taq* polymerase, 1 µM of each

primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer, 1X Q-Solution (reagents by Qiagen) and 1 µl of a 1: 10 dilution of genomic DNA as template. The second round of nested PCR used Primer 3 and Primer 4 (5'- GGC CAC GCG TCG ACT AGT AC-3'). Cycling conditions were as follows: 95°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 42°C for 35 seconds and 72°C for 45 seconds and a final single step of 72°C for 5 minutes. Each 50 µl PCR reaction contained 2.5 U of *Taq* polymerase, 1 µM of each primer, 200 µM of each dNTP, 1X PCR buffer, 1X Q-Solution (reagents by Qiagen) and 3 µl of PCR product from the first round of PCR as template. Product from the second round of PCR was purified using a QIAquick PCR purification kit (Qiagen) and visualised by electrophoresis on a 2% agarose gel. DNA was quantified using a Nanodrop 1000 (Thermofisher Scientific). In order to identify DNA flanking the transposon insertion site amplicons were sequenced using Primer 3. Transposon insertions were mapped to the *B. lata* strain 383 genome (www.JGI.doe.gov) by conducting a database search, using the Basic alignment search tool (BLAST), at the *Burkholderia* Genome Database (www.Burkholderia.com).

2.11 ASSESSING BCC SPECIES DIVERSITY IN THE ENVIRONMENTAL INDUSTRIAL NICHE

2.11.1 Multi locus sequence typing (MLST) analysis of a collection of Bcc isolates from environmental industrial sources.

Multi locus sequence typing, a highly discriminatory strain typing method described by Baldwin *et al.* (2005), was performed on a collection of 67 Bcc isolated from environmental industrial sources by Dr. Adam Baldwin (unpublished). Sources included aircraft fuel containers, water filter-heads, pharmaceutical products, raw materials used in industrial processes and aqueous product. Environmental industrial sources were from various geographical locations, including Europe, Australasia, Asia, North and South America. The MLST website (http:pubmlst.org/bcc/) developed by Jolley *et al.*(2004) was used to match allelic profiles and determine sequence types (ST) where appropriate; generation of a ST number required the allelic profiling of all seven housekeeping genes, whereas identification to the species level could be achieved by profiling $\geq$2 of the housekeeping genes. A selection of 19 environmental industrial Bcc strains, identified by MLST analysis, was included in the strain collections used to evaluate Bcc preservative susceptibility (see section 2.12).

2.12 PRESERVATIVE SUCEPTIBILITY PROFILING

2.12.1 Minimum inhibitory concentrations (MIC) of individual preservatives and preservative enhancers

2.12.1.1 Broth dilution assay

As a preliminary investigation, the susceptibility of eight Bcc strains (Table 5), representing prevalent species groups in a collection of industrial isolates (see section 2.11.1), to preservatives and preservative enhancers (Table 2) was determined by broth and agar dilution methods. The eight Bcc strains were then included in the larger collection of 83 Bcc used in subsequent preservative susceptibility testing (see section 2.12.3). Three non-Bcc bacteria were included in preliminary investigations of preservative susceptibility: antibiotic reference strains *E. coli* (ATCC 12241), *S. aureus* (ATCC 12981) and *P. aeruginosa* (ATCC 12903). Non-Bcc bacteria were assayed with the highest and lowest MIC of the eight Bcc evaluated. Stock solutions of preservative or preservative enhancer were added to BSM (CYG) broth to achieve final concentrations ranging from: 0 – 0.001213% v/v methylisothiazolinone, 0 – 0.0006% v/v isothiazolinone blend, 0 – 0.002% w/v benzisothiazolinone, 0 – 0.1% w/v benzethonium chloride, 0 – 0.81% v/v DMDM hydantoin, 0- 0.15% v/v phenoxyethanol, 0- 0.2% w/v methyl paraben, 0 - 0.04% w/v propyl paraben, 0- 0.75% w/v sodium benzoate and 0 – 5% EDTA .

Bcc strains (except *B. cenocepacia* J2315) were cultured for 18 hours in 3 ml of BSM (CYG) broth at 30ºC in a 14 ml tube shaken horizontally at 150 rpm; *B. cenocepacia* J2315 and non-Bcc reference strains were cultured at 37°C. Cultures were adjusted to an OD of 1±0.2 at 600 nm, equating to approximately 1 x 10^8 CFU/ml. Approximately 1 x 10^5 CFU of the test strains was added to the test and control BSM (CYG) media (no preservative or preservative enhancer) in replicate wells of a 96-well microtitre plate (200 µl). Microplates were incubated with shaking (200 rpm) for 24 hours at 30ºC or 37°C depending on the test organism. The MIC was designated as the concentration of preservative at which there was an 80% reduction in optical density from the average turbidity reading of control wells at 630 nm. Experiments were repeated with different starting cultures to obtain biological replicates.

2.12.1.2 Agar dilution assay

The restricted solubility of propyl paraben (>0.04%) and the opacity of caprylyl glycol and Ethylhexyl gylcerin prevented the evaluation of a range of concentrations in BSM (CYG) broth. An agar dilution assay was used to investigate susceptibility to these agents, as follows: after autoclaving, the BSM (CYG) agar was cooled to 50ºC and an appropriate volume of preservative stock added to the 25 ml of agar to achieve final concentration ranges of 0 – 0.1% w/v for propyl paraben, 0 – 0.4% w/v caprylyl glycol, and 0 – 2% w/v ethylhexyl glycerin; Bcc strains were cultured for 18 hours as previously described (section 2.12.1.1) and diluted to an OD of 1±0.2 (600 nm); triplicate 10 µl drops of bacterial suspension were aspirated onto the surface of BSM (CYG) agar containing preservative or preservative enhancer and control BSM (CYG) agar plates without preservative or enhancers. Plates were incubated for 24 hours at 30 or 37°C depending on the test organism. The MIC was defined as the minimum concentration of agent required to inhibit bacterial growth.

Table 5. The eight Bcc strains used in preliminary preservative susceptibility testing

Species, Strain name	Accession number from BCCM/LMG culture collections (Other strain designations)	Isolation source and other information	Isolation source code	MLST Sequence type
B. arboris				
BCC1306	-	Environmental industrial	ENVI	325 [1]
B. cenocepacia III-A				
J2315 [ESP]	LMG 16656 [T]	Cystic fibrosis patient sputum, UK; genome available (www.sanger.ac.uk/Projects/B_cenocepacia/)	CLIN	28
B. cenocepacia III-B				
BCC1283	-	Environmental industrial	ENVI	250 [1]
HI2424	-	Soil, USA; genome available (http://genome.jgi-psf.org/mic_cur1.html)	ENV	122
BCC1291	-	Environmental industrial	ENVI	322 [1]
B. lata				
Strain 383	LMG 22485 [T]	Forest soil, Trinidad; genome available (http://genome.jgi-psf.org/bur94/bur94.home.html)	ENV	101
BCC1294	-	Environmental industrial	ENVI	98 [1]
BCC1296	-	Environmental industrial	ENVI	119 [1]

Footnotes:

BCCM/LMG, Belgian co-ordinated collections of micro-organisms, Ghent.
BCC, Cardiff strain collection
CLIN, clinical; ENV, environmental; ENVI, environmental industrial.
MLST, multi locus sequence typing
[1] Strain included in MLST analysis of Bcc isolates from environmental industrial sources (this study)
[T] Type strain
[ESP] *Burkholderia cepacia* complex experimental strain panel

2.12.2 Preservative combination testing

A checkerboard method was used for the measurement of susceptibility to combined preservatives and preservatives with enhancers as described by Denyer *et al*.(1985). Checkerboard assays involved exposing *B. lata* strain 383 (LMG22485) to serial dilutions of two agents in BSM (CYG) broth. The blend of methylisothiazolinone and chloromethylisothiazolinone preservatives was considered as just one agent for the purpose of this assay.

2.12.2.1 Checkerboard method

The range of concentrations evaluated extended just above the expected minimum inhibitory concentration for each agent to zero. Samples were arranged so that the test organism was exposed to all possible combinations of test concentrations; the primary preservative serial dilutions increasing (1.5-fold) across the horizontal axis; the secondary preservative or preservative enhancer serial dilutions increasing (1.5-fold) on the vertical axis of a microtitre plate (Fisher). Methylisothiazolinone and the blend of isothiazolinone preservatives were evaluated as primary agents in combination with the following secondary agents: methyl paraben, phenoxyethanol, DMDM hydantoin and EDTA. As an additional control, the primary agent was also assayed in combination with itself. Appropriate amounts of stock solutions were added to BSM (CYG) broth to achieve double that of the desired final concentration for the primary and secondary agents. Serial dilutions increased 1.5-fold, final test concentrations ranged from: 0 – 0.00194% v/v for methylisothiazolinone, 0 – 0.0000749% v/v for the isothiazolinone preservative blend , 0 – 0.004% w/v for benzisothiazolinone, 0 – 0.0081% v/v DMDM hydantoin, 0 – 0.15% w/v methyl paraben, 0 – 0.02% w/v benzethonium chloride, 0 – 0.3% v/v phenoxyethanol and 0 – 6% v/v EDTA. Ethylhexyl glycerin was not evaluated as its opacity in solution prevented turbidometric analysis.

B. lata strain 383 was cultured for 18 hours as previously described and diluted to an OD of 1±0.2 (600 nm). Approximately $5x10^5$ CFU were added to 1 ml of the secondary agent at double the desired test concentration. Immediately, 100 µl of the suspension was combined with 100 µl of the primary agent in a well of a microtitre plate, thus diluting each agent to its desired final concentration. Exposure to double the desired concentration

of the secondary agent for ≤ 10 minutes did not alter viability. Plates were incubated at 30°C, with shaking at 150 rpm for 24 hours. The MIC was designated as the concentration of preservative at which there was an 80% reduction in optical density from the average turbidity reading of the control well (BSM (CYG) broth without preservative or enhancing agent) at 630 nm. The combination first showing an inhibition of growth was taken as the end-point.

2.12.2.2 Bioscreen C assay

A checkerboard approach in a Bioscreen C Microbiological Growth analyser (Labsystems, Finland) was adopted to examine the growth dynamics of *B. lata* strain 383 cultured in BSM (CYG) broth containing the following combinations of preservative agents: methylisothiazolinone in combination with methyl paraben and the isothiazolinone blend in combination with phenoxyethanol. Appropriate amounts of stock solutions were added to BSM (CYG) broth to achieve double that of the desired final concentration for the primary and secondary agents. Serial dilutions (1.5-fold) were made and final test concentrations ranged from: 0 – 0.001455% v/v methylisothiazolinone, 0 – 0.113% w/v methyl paraben, 0 – 0.000056% v/v isothiazolinone preservative blend and 0 – 0.225% v/v phenoxyethanol. The inoculum was prepared as previously described in section 2.12.2.1 above. 100 µl of the secondary preservative agent containing the inoculum was immediately transferred to duplicate wells of the Bioscreen microplate containing the primary test agent (100 µl). Growth analysis was performed for 48 hours at 30°C; turbidity readings were taken at 5 minute intervals using a wide-band filter (450-580nm) after shaking the microplates for 10 seconds at a medium intensity. The MIC was designated as the lowest concentration of preservative at which there was an 80% reduction in optical density from the average turbidity reading of the control wells (BSM (CYG) broth without preservative or enhancing agent).

2.12.2.3 Interpretation of checkerboard synergy testing

The observation of synergy between two agents, defined as activity beyond that of the additive effect of individual agents, involves analysis of their individual MIC and the combined MIC for the test organism. The simultaneous activity of two agents in combination was obtained from the sum of the fractional inhibitory concentration (FIC) of each agent using the following equation:

$$FIC = \frac{\text{MIC of preservative in combination}}{\text{MIC of preservative alone}}$$

$\sum$FIC = FIC of primary preservative + FIC of secondary preservative

A synergistic outcome was defined as an $\sum FIC \leq 0.5$, additivity/indifference was defined as an $\sum FIC > 0.5$, antagonism was defined as an $\sum FIC > 4$. The lowest FIC index of all the non-turbid wells along the turbidity/non-turbidity interface was used.

2.12.3 Preservative susceptibility of a collection of 83 Bcc bacteria

A collection of 83 genetically distinct Bcc strains selected from the Mahenthiralingam Group collection at Cardiff University and Belgium coordinated Collection of Microorganisms (BCCM: http://bccm.belspo.be/about/lmg.php) were used to investigate the relationship between preservative susceptibility, Bcc species diversity and isolation source Table 6. Bcc strains, identified and typed by MLST (Baldwin *et al.*, 2005), spanning the genetically diverse 17 species groups (Vanlaere *et al.*, 2009), five novel Bcc groups (recently resolved by MLST schemes) and unclassified novel Bcc. The selected strains encompassed reference strains from the *Burkholderia cepacia* complex experimental strain panel (Coenye *et al.*, 2003; Mahenthiralingam *et al.*, 2000b), Type strains, and typical preservative challenge test organisms (Orth *et al*, 2006). Strains varied in isolation source and included clinical (41), environmental (24) and environmental industrial (20) strains. Ten non-Bcc species evaluated as a control group included multi drug resistant reference strains, clinical and industrial isolates and represented typical preservative challenge test organisms (Table 7).

2.12.3.1 MIC determination by agar dilution assay

An agar dilution-based assay was used to determine the susceptibility of the collection of strains to seven preservative agents. Aqueous stock solutions (10% v/v) of dimethylol dimethyl hydantoin, methylisothiazolinone, isothiazolinone preservative blend, and (10% w/v) benzisothiazolinone, benzethonium chloride, and sodium benzoate were made. Due to the restricted solubility of methyl paraben and phenoxyethanol, stock solutions (10% w/v and v/v respectively) were prepared in DMSO. Final concentrations of DMSO in the presence of the bacteria did not exceed 1% v/v and was non-toxic to the test organisms.

After autoclaving, the BSM (CYG) agar was cooled to 50ºC and an appropriate volume of preservative stock added to the 40 ml of agar to achieve final concentration ranges of 0 – 0.008% w/v for benzisothiazolinone, 0 – 0.4% w/v benzethonium chloride, 0 – 0.216% v/v dimethylol dimethyl hydantoin, 0 – 0.004365% v/v methylisothiazolinone, 0 - 0.000674% v/v methylisothiazolinone /chloromethylisothiazolinone, 0 – 0.4% w/v methyl paraben, 0.3% v/v phenoxyethanol and 0 – 0.8% w/v sodium benzoate. The triple vented 120 mm square petri dishes (International Scientific supplies Ltd, UK) were then over-dried. To avoid a reduction in the activity of sodium benzoate the pH of the media was adjusted from pH 7 to pH 5 for that particular evaluation only. Master plates of the bacterial collection were produced as follows: strains were cultured for 18 hours in 3 ml of BSM (CYG) broth at 30ºC as previously described; cultures were adjusted to an OD of 1±0.2 (600 nm), DMSO was added as a cryoprotectant (8% v/v) and each suspension transferred to a designated well of a 96 well plate (Fisher Scientific) which was then stored at – 80ºC. A new master plate was defrosted for 2 hours for the evaluation of each preservative agent. Once defrosted a multi point inoculator was used to transfer approximately 1.5 µl of each strain to a 96 well microplate containing BSM (CYG) broth (200 µl per well). The micro plates were incubated with shaking (150 rpm) at 30ºC for 18 hours and the OD was read using a Dynex Technologies MRX® microplate absorbance reader with Revalation application. Strains reached a consistent OD when revived by this method which correlated to viable counts of between ~$1x10^8$ and ~$1x10^9$ CFU/ml. A multi point inoculator was used to place approximately 1.5 µl spots of each culture from the revival plate onto the agar surface of triplicate test and control plates. Plates were incubated at 30ºC for 24 hours. The MIC was designated as the lowest concentration of preservative at which no there was no visible growth of the test organism.

2.12.4 Minimum bactericidal concentrations (MBC)

2.12.4.1 Neutraliser efficiency testing

An efficient means of terminating or quenching the activity of preservatives, which was non-toxic to the test organisms, was required for MBC testing. Neutralising solutions, recommended by Unilever (2% w/v Tween 80 and 0.1% w/v peptone), and described by Lear *et al*. (2006) (5% w/v Tween 80, 1.5% w/v lecithin) were evaluated prior to MBC experimentation. *B. lata* strain 383, cultured for 18 hours as previously described, was

diluted to an OD of 1±0.2 (600 nm). To evaluate toxicity of the neutralising solutions, 100 µl of a 1x 10^6 CFU/ml suspension was transferred to 900 µl of neutraliser and dH_2O as a control, and incubated statically at 20°C for 10 minutes. Viable cells were enumerated as described in section 2.4.3. To determine the efficiency of inactivation of preservatives, *B. lata* strain 383 was exposed to the following putatively lethal preservative concentrations: 0.097% v/v methylisothiazolinone, 0.01498% v/v for the isothiazolinone preservative blend, 0.2% w/v benzisothiazolinone, 0.5% w/v benzethonium chloride, 2% v/v phenoxyethanol, 0.54% v/v DMDM hydantoin, 0.2% w/v methyl paraben and 1% w/v sodium benzoate. 100 µl of preservative at a putative lethal concentration was added to 800 µl of neutraliser, or dH_2O as control, immediately followed by 100 µl of a 1x 10^6 CFU/ml bacterial suspension and incubated statically at 20°C for 10 minutes. Viable cells were enumerated on BSM (CYG) agar as described in section 2.4.3. The neutralising solution - 2% w/v Tween 80 and 0.1% w/v peptone, in conjunction with dilution of the preservative- were the chosen methods of inactivating preservative activity in MBC experiments.

2.12.4.2 MBC assay

Preservative concentrations evaluated ranged from the maximum permitted concentrations for use in rinse-off personal care products in EU regulated countries (Directive 76/768/EEC) to zero. Additional concentrations up to 10 fold, and 2 fold higher than the maximum permitted levels of benzethonium chloride and DMDM hydantoin respectively were also evaluated. Concentrations up to 5 fold higher than the maximum permitted levels of phenoxyethanol and sodium benzoate were also evaluated. The maximum concentration of benzisothiazolinone (0.015% w/v) evaluated was based on the manufacturers' recommendation; this agent is not permitted for use in personal care products in EU regulated countries. Maximum permitted levels are as follows: 0.1% w/v benzethonium chloride, 0.3% v/v dimethylol dimethyl hydantoin, 0.01% v/v methylisothiazolinone, 0.0015% v/v isothiazolinone preservative blend, 1% v/v phenoxyethanol and 0.5% w/v sodium benzoate. Due to solubility issues methyl paraben could not be evaluated at higher bactericidal concentrations (>0.2% w/v) using this method.

A master plate of the collection of 83 Bcc strains (Table 6) and 10 non-Bcc strains (Table 7) was defrosted and revived in BSM (CYG) broth as described in section 2.12.3.1. The revived cultures were diluted tenfold and 20 µl of the each strain transferred to duplicate microplates with test preservative concentrations and duplicate control plates without preservative; test plates and one control plate were incubated with shaking (150 rpm) at 30ºC for 24 hours. To ensure the starting inoculum was of a detectable size, one control plate was immediately neutralised and replica plated onto agar without overnight incubation in the microtitre plate. After incubation of the microtitre test plates, 20 µl of the bacterial suspension was placed into 180 µl of neutraliser solution (also diluted the preservative concentration tenfold) and left in contact for 10 minutes. After neutralisation, a multi-point inoculator was used to place approximately 1.5 µl spots of each strain onto the surface of triplicate BSM (CYG) agar plates and incubated for 24-72 hours at 30ºC; the MBC was determined as the lowest concentration at which growth ceased. As the starting number of viable cells was known it was calculated that this method was capable of detecting a ~99.25% rate of bacterial kill. To improve the efficacy of neutralisation of higher test concentrations, after neutralisation of concentrations at or above the maximum permitted level, a multi point inoculator was used to inoculate a 96-well microtitre plate with 200 µl BSM (CYG) per well. The enrichment plates were incubated with shaking (150 rpm) at 30ºC for 24 hours, and a visually assessment of growth conducted. A multi point inoculator was used to place approximately 1.5 µl spots of each strain onto the surface of triplicate BSM (CYG) agar plates to identify viable cells.

Table 6. Collection of 83 Bcc strains used in preservative susceptibility testing

Species, Strain name	Accession number from BCCM/LMG culture collection (Other strain designations)	Isolation source and other information	Isolation source code	MLST Sequence type
B. ambifaria				
AMMD [ESP]	LMG 19182 [T]	Pea rhizosphere, USA; genome available (http://genome.jgi-psf.org/mic_cur1.html)	ENV	77
BCC0267 [ESP]	LMG 19467	Cystic fibrosis patient	CLIN	78
BCC0338	LMG 17828	Corn rhizosphere, USA	ENV	74
MEX-5	-	genome avaliable (http://genome.jgi-psf.org/mic_cur1.html)	ENV	-
B. anthina				
BCC0635 [ESP]	LMG 16670	Carludovica palmata, rhizosphere, UK.	ENV	89
BCC0639 [ESP]	LMG 20980 [T]	Soil, USA	ENV	86
B. arboris				
BCC0049	-	Clinical isolate, Europe	CLIN	110
BCC1306	-	Environmental industrial	ENVI	325 [1]
BCC1310	-	Environmental Industrial	ENVI	327 [1]
BCC1312	-	Environmental industrial	ENVI	328 [1]
B. cenocepacia III-A				
BCC0016	LMG18827	Cystic fibrosis patient sputum, Canada	CLIN	29
BCC0018 [ESP]	LMG 16659	Cystic fibrosis patient ,UK	CLIN	35
BCC0272	-	Cystic fibrosis patient sputum, Canada	CLIN	31
BCC0560	-	Cystic fibrosis patient , Canada	CLIN	33
J2315 [ESP]	LMG 16656 [T]	Cystic fibrosis patient sputum, UK; genome available (www.sanger.ac.uk/Projects/B_cenocepacia/)	CLIN	28
BCC0742	(X100)	Cystic fibrosis patient sputum, Prague	CLIN	32
K56-2	-	Cystic fibrosis patient	CLIN	30
B. cenocepacia III-B				
BCC0019 [ESP]	LMG 18829	Cystic fibrosis patient, USA	CLIN	40
BCC0020 [ESP]	LMG 18830	Cystic fibrosis patient, Australia	CLIN	39
BCC0021 [ESP]	LMG 16654	Cystic fibrosis patient, UK	CLIN	34
BCC0022 [ESP]	LMG 18832	Urinary tract infection,UK	CLIN	36
AU1054	-	Cystic fibrosis patient, USA; genome available (http://genome.jgi-psf.org/mic_cur1.html)	CLIN	122
HI2424	-	Soil, USA; genome available (http://genome.jgi-psf.org/mic_cur1.html)	ENV	122
BCC1283	-	Environmental industrial	ENVI	250 [1]
BCC1291	-	Environmental industrial	ENVI	322 [1]
B. cenocepacia III-D				
BCC0446	-	Cystic fibrosis patient, Sicily	CLIN	46
B. cepacia				
BCC0001 [ESP]	LMG 1222 [T]	Onion rot, USA	ENV	10
BCC0002 [ESP]	LMG 2161	Forest soil, Trinidad	ENV	1
BCC0003 [ESP]	LMG 18821	Cystic fibrosis patient, Australia	CLIN	5
BCC0004 [ESP]	LMG 17997	Urinary tract infection, Sweden	CLIN	2
B. contaminans				
BCC0362	(R-9929)	Cystic fibrosis patient	CLIN	97
SAR-1	LMG 23255	Cystic fibrosis patient, Czech Republic; metagenomic strain from sargasso sea	CLIN	102
BCC1281	LMG 23254	seawater enriched with polyhyfroxubutyrate, Japan	ENV	178
BCC1315	-	Environmental industrial	ENVI	341 [1]
BCC1323	-	Environmental industrial	ENVI	323 [1]
B. diffusa				
BCC0106	LMG 24266	Cystic fibrosis patient's throat, Canada	CLIN	107
BCC0169	(ATCC 29352)	Soil	ENV	108
AU1075	LMG 24065	Cystic fibrosis patient, USA	CLIN	164
B. dolosa				
BCC0072	(R-2879)	Cystic fibrosis patient, USA	CLIN	72
AU0645 [ESP]	LMG 18943 [T]	Cystic fibrosis patient, USA	CLIN	72
AU3556	-	Cystic fibrosis patient	CLIN	215

Table 6. Collection of 83 Bcc strains used in preservative susceptibility testing (continued)

Species, Strain name	Accession number from BCCM/LMG culture collection (Other strain designations)	Isolation source and other information	Isolation source code	MLST Sequence type
B. lata				
Strain 383	LMG 22485 ^{T}	Forest soil, Trinidad; genome available (http://genome.jgi-psf.org/bur94/bur94.home.html)	ENV	101
BCC1294	-	Environmental industrial	ENVI	98 [1]
BCC1296	-	Environmental industrial	ENVI	119 [1]
BCC1321	-	Environmental industrial	ENVI	339 [1]
BCC1406	-	Environmental industrial	ENVI	103 [1]
B. metallica				
BCC0095	-	Cystic fibrosis patient sputum,Canada	CLIN	288
AU0553	LMG 24068 ^{T}	Cystic fibrosis patient,USA	CLIN	-
B. multivorans				
BCC0005 ^{ESP}	LMG 18822	Cystic fibrosis patient, Canada	CLIN	19
BCC0008 ^{ESP}	LMG 16660	Cystic fibrosis patient, UK	CLIN	27
ATCC17616 ^{ESP}	LMG 17588	Soil,US ; genome available	ENV	21
BCC0390 ^{ESP}	LMG 18825	Cystic fibrosis patient, UK	CLIN	15
BCC0764 ^{ESP}	LMG 13010 ^{T}	Cystic fibrosis patient, Belgium	CLIN	397
BCC1421 ^{ESP}	LMG 16660	Cystic fibrosis patient, UK	CLIN	-
BCC1430	-	Cystic fibrosis patient, UK	CLIN	-
BCC1560	-	Environmental industrial	ENVI	439
B. pyrrocinia				
BCC0171 ^{ESP}	LMG 21822	Corn field soil,USA	ENV	95
BCC0180 $^{T\ ESP}$	LMG 14191 ^{T}	Soil	ENV	41
BCC0476 ^{ESP}	LMG 21823	Water,UK	ENV	92
B. stabilis				
BCC0023 ^{ESP}	LMG 14294 ^{T}	Cystic fibrosis patient sputum,Belgium	CLIN	50
BCC0286	(ATCC 35254)	Povidone-iodine solution,USA	ENV [a]	51
AU6735	-	Cystic fibrosis patient	CLIN	53
B. ubonensis				
BCC1603	LMG 20358	Surface soil,Thailand	ENV	-
B. vietnamiensis				
BCC0027 ^{ESP}	LMG 18835	Cystic fibrosis patient, USA	CLIN	58
BCC0028 ^{ESP}	LMG 16232	Cystic fibrosis patient sputum, Sweden	CLIN	200
BCC0030 ^{ESP}	LMG 10929 ^{T}	rice rhizosphere soil,Vietnam	ENV	65
BCC1304	-	Environmental industrial	ENVI	-
BCC1309	-	Environmental industrial	ENVI	326

Table 6. Collection of 83 Bcc strains used in preservative susceptibility testing (continued)

Species, Strain name	Accession number from BCCM/LMG culture collection (Other strain designations)	Isolation source and other information	Isolation source code	MLST Sequence type
BCC Kc				
BCC1282	-	Environmental industrial	ENVI	333 [1]
BCC1300	-	Environmental industrial	ENVI	334 [1]
BCC1313	-	Environmental industrial	ENVI	335 [1]
BCC1314	-	Environmental industrial	ENVI	336 [1]
BCC4				
BCC0322 [ESP]	LMG 21824	Cystic fibrosis patient, USA	CLIN	113
BCC0405	(T21)	Environment	ENV	112
BCC0475	-	Environment	ENV	114
BCC5				
BCC0397	(R-10733t1)	Clinincal sample	CLIN	207
BCC0474	-	Cystic fibrosis patient, Italy	CLIN	185
BCC1017	-	Environment	ENV	140
BCC6				
BCC0044	-	Cystic fibrosis patient,UK	CLIN	206
BCC0288	-	Cystic fibrosis patient,UK	CLIN	116
BCC0398	(R-10741)	Environment	ENV	127
BCC8				
BCC1191	-	Cystic fibrosis patient	ClIN	307
unclassified BCC				
BCC0198	(R-6622)	Environment	ENV	48
BCC0276	-	Soil, USA	ENV	47

Footnotes:

BCCM/LMG, Belgian co-ordinated collections of micro-organisms, Ghent.
ATCC, American type culture collection
BCC, Cardiff strain collection
CLIN, clinical ; ENV, environmental ; ENVI, environmental industrial.
MLST, Multi locus sequence typing
[1] Strain included in MLST study of Bcc isolates from environmental industrial sources
[T] Type strain
[ESP] *Burkholderia cepacia* complex experimental strain panel
[a] Hospital environmental isolate

Table 7. Non-Bcc bacteria used in preservative susceptibility testing

Species, Accession number	Isolation source	Isolation source code
Pandoraea pnomenusa BCC0092	Cystic fibrosis patient sputum,Canada	CLIN
Pandoraea sputorum BCC0212	Cystic fibrosis patient sputum,Canada	CLIN
Acinetobacter baumanni BCC0810	Clinical sample	CLIN
Burkholderia gladioli [1] BCC1317	Environmental Industrial	ENVI
Pseudomonas fluorescens LMG 1794 [T]	pre-filter water-works tanks,UK	ENV
Pseudomonas putida LMG 2257 [T]	Soil, USA	ENV
Ralstonia mannitolytica LMG 6866 [T]	Contaminated autoclave fluid,UK [a]	ENV
Ralstonia pickettii LMG 5942 [T]	Tracheotomy patient, USA	CLIN
Stenotrophomonas maltophilia LMG 958 [T]	Mouth cancer patient	CLIN
Enterococcus feacalis ATCC 51299	Peritoneal fluid,USA	CLIN

Footnotes:

LMG, BCCM/LMG culture collection, Ghent.
ATCC, American type culture collection
BCC, Cardiff strain collection
CLIN, clinical ; ENV, environmental ; ENVI, environmental industrial.
[1] Strain included in MLST study of Bcc isolates from environmental industrial sources
[T] Type strain
[a] Source of outbreak infection in hospital

2.13 ADAPTIVE RESISTANCE TO PRESERVATIVES

MLST analysis indicated *B. lata* to be a commonly encountered species in a collection of industrial isolates (see section 3.2.1). Type strain *B. lata* 383 (LMG 22485), with available genome sequence (http://www.jgi.doe.gov/) and mid to low ranging preservative tolerance, was used as a model strain to evaluate adaptive Bcc resistance to preservatives.

2.13.1 Agar dilution step-wise training of *B. lata* strain 383

An agar dilution method was used to evaluate adaptive resistance to preservatives. Strain 383 was cultured for 18 hours in 3 ml of BSM (CYG) broth at 30°C as previously described. To verify the MIC of the parental strain, ~1 x 10^6 CFU of *B. lata* strain 383 were inoculated onto the surface of BSM (CYG) agar plates containing a range of preservative concentrations; plates were incubated for 24 hours at 30°C and the MIC was designated as the lowest concentration of preservative at which no there was no visible growth. BSM (CYG) agar plates with a range of preservative concentrations of ≤ fourfold lower than the MIC of the parental strain were prepared and inoculated with 1 x 10^6 CFU; plates were incubated for 24 - 72 hours at 30°C. Approximately two to three colonies swabbed from the BSM (CYG) agar with the highest preservative concentration in the range were used to inoculate the next set of training plates; each plate with an increased concentration (1.5 or 2 fold dependent on assay) of the preservative. If no growth was apparent after 96 hours, the increase in preservative concentration was lowered, and fresh plates were inoculated with growth from the previous training plate. Stepwise training was stopped when subculture onto an increased concentration did not result in growth within 168 hours. Derivatives of *B. lata* strain 383 cultured on BSM (CYG) agar with preservative concentrations above the wild-type MIC were stored at - 80°C in BSM (CYG) broth, with 8% v/v DMSO as a cryoprotectant for further analysis. After stepwise training, the identity of the derivatives was confirmed as *B. lata* strain 383 by RAPD analysis (section 2.6) and each was renamed to reflect the preservative to which it had been exposed to during stepwise training.

2.13.2 Assessing the phenotypic stability of derivatives

Stability of the adaptation was confirmed by repeated subculture (x10) on BSM (CYG) in the absence of preservative. Identity of the derivatives was confirmed by RAPD analysis

(Section 2.6) after repeated subculture. The antibiotic and preservative susceptibility of the passaged derivatives was determined as described in sections 2.13.4.1 and 2.13.2.1. Passaged derivatives were stored at - 80°C in BSM (CYG) broth with 8% v/v DMSO as a cryoprotectant for further analysis.

2.13.2.1 Preservative MIC by broth dilution assay

A modified broth microdilution assay, based on Clinical and Laboratory Standard Institute (CLSI) methodology, was used to determine the preservative MICs of the *B. lata* 383 parental strain and derivatives before and after repeated subculture in the absence of preservative. BSM (CYG) broth with preservative concentrations ranging from 0 – 0.2% w/v for benzisothiazolinone, 0 – 0.05% w/v benzethonium chloride, 0 – 0.405% v/v dimethylol dimethyl hydantoin, 0 – 0.097% v/v methylisothiazolinone, 0 - 0.000749% v/v isothiazolinone blend, 0 – 0.2% w/v methyl paraben, and 0 - 0.75% v/v phenoxyethanol were evaluated. Sodium benzoate was not evaluated as growth dynamics of the derivatives at pH 5 had not been investigated. 18- hour cultures were prepared in BSM (CYG) broth as previously described and diluted to an OD of 1±0.2 (600nm); this consistently equated to ~ 5 x 10^8 CFU/ml for this strain. 100 µl of a 5 x 10^5 CFU/ml suspension of the test strain was added to 100 µl of the test media at double the desired concentration (diluting it to the desired final test concentration), and control broth without preservative, in triplicate wells of a microtitre plate (200 µl). Microtire plates were incubated with shaking (150 rpm) at 30ºC for 24 hours and the MIC designated as the concentration of preservative at which there was no visible bacterial growth.

2.13.3 Growth analysis of *B. lata* strain 383 derivatives cultured in the presence of preservative using the Bioscreen C MBR

The growth dynamics of *B. lata* strain 383 wild-type and its derivatives were examined using an adapted broth dilution assay in a Bioscreen Microbiolgical Growth analyser (Labsystems, Finland). Preservative concentrations evaluated were as follows: 0 - 0.1% w/v benzethonium chloride, 0 - 0.02% w/v benzisothiazolinone, 0 - 0.000375% v/v isothiazolinone blend, 0 - 0.0405% v/v DMDM hydantoin, 0 - 0.0097% v/v methylisothiazolinone, 0 - 0.1875% w/v methyl paraben and 0 - 1.5% v/v phenoxyethanol; sodium benzoate was not evaluated. Strain 383 and derivatives were cultured as previously described. Cultures were diluted to an OD of 1±0.2 (600nm); test

and control media were inoculated with approximately 1 x 10^5 CFU/ml and transferred to triplicate wells of the Bioscreen microplate (200 µl). Growth analysis was performed for 72 hours at 30°C; turbidity measurements were taken at 5 minute intervals using a wide-band filter (450-580 nm), after shaking the microplates for 10 seconds at an intermediate intensity. Experiments were repeated with different starting cultures to obtain two biological replicates, there being six technical replicates in total.

2.13.3.1 Estimation of lag phase and growth rate

The mean optical density of triplicate test wells (after subtraction of the mean optical density of blank wells without inoculum) were used to generate growth curves. Estimations of the length of lag phase (hours) and growth rate (µ) of the parental *B. lata* strain and derivatives, cultured in the presence and absence of preservatives, were determined from the growth curves generated. The growth rate was calculated as follows:

$$\mu = (ln N_t - ln N_o) / (t - t_0)$$

Where N_t is the optical density at time $_t$ and N_o is the optical density at time t_0 .

2.13.4 Antibiotic susceptibility profiling of *B. lata* strain 383 and derivatives

2.13.4.1 Antibiotic ETests®

The antibiotic susceptibility of *B. lata* 383 and its derivatives was determined using Etest® strips as described by the manufacturer (AB Biodisk). Nine antibiotics were examined: amikacin, azithromycin, ceftazidime, ciprofloxacin, chloramphenicol, piperacillin, trimethoprim/sulfamethoxazole, piperacillin and meropenem. These represented six classes of antibiotic with varying cellular targets (Chopra, 1996), all of which may be used in the treatment of microbial infection in individuals with CF (Rose *et al.*, 2009). Three antibiotic reference organisms, *S. aureus* (NTCC 12981), *P. aeruginosa* (NTCC 12903) and *E. coli* (NTCC 12241) were included as controls. *B. lata* strain 383 and derivatives were revived on BSM (CYG) agar plates, reference strains on LB agar, and fresh colony growth was resuspended in Iso-sensitest broth (Oxoid, Basingstoke, UK) to an OD of 0.5 at 630 nm. Each bacterial suspension was swabbed onto duplicate Iso-sensitest agar plates and left to dry for 15 minutes. Etest® strips were placed on the surface of the lawn of bacteria and

incubated for 24 hours at 30°C or 37°C for reference strains; the MIC was determined using the manufacturer's guidelines. Experiments were repeated with different starting cultures to obtain biological replicates. An overall antibiotic susceptibility profile score for the wild-type and derivatives was calculated by averaging the MICs. This was used as a measure of overall multi-drug resistance in the *B. lata* wild-type and derivatives (Rose *et al.*, 2009).

2.13.4.2 Fluoroquinolone susceptibility profiling of *B. lata* 383 derivatives

A modified broth microdilution assay, based on CLSI guidelines, was used to further evaluate the susceptibility of the parental *B. lata* strain 383 and derivatives to fluoroquinolone antibiotics. Aqueous solutions of fluoroquinolone antibiotics (10 mg/ml) were added to cation-adjusted Mueller Hinton (MH) broth to make concentrations ranging from 0 – 80 µg/ml for ciprofloxacin, 0 – 256 µg/ml for levofloxacin, 0 – 1024 µg/ml for norfloxacin and 0 – 256 µg/ml for sparfloxacin. *B. lata* and derivatives were cultured for 18 h in BSM (CYG) broth as previously described. Cultures were diluted to an OD of 1±0.2 (600 nm) in cation-adjusted MH broth, equating to approximatley 5 x 10^8 CFU/ml. Approximately 1 x 10^5 CFU of the test organism were added to the test and control media in triplicate wells of a 96 well microtitre plate (200 µl). Microplates were incubated with shaking (150 rpm) at 30ºC for 24 hours; the MIC was designated as the concentration of antibiotic at which there was no visible bacterial growth. To determine the MBC, 100 µl from the first four clear wells was aspirated onto a single antibiotic free agar plate where it was allowed to dry partially and then spread over the surface of the agar. The MBC was determined as the concentration of antibiotic which achieved a 99.9% or 3 $\log_{10}$ reduction in viable cells.

2.13.4.3 Screening for mutations in the topoisomerase genes of *B. lata* derivatives

The main mechanism of fluoroquinolone resistance in Gram negative bacteria develops by accumulation of mutations in the quinolone resistance-determining region (QRDR) of the topoisomerase genes (Drlica & Malik, 2003; Pope *et al.*, 2008). The QRDR of *gyrA, gyrB* genes and an extended region (that encompassed the QRDR) of the *parC* and *parE* genes were sequenced in the *B. lata* 383 parental strain and derivatives using the primers listed (Table 8)

The amplification conditions were as follows: DNA was denatured at 96ºC for 1 minute, followed by 30 cycles of denaturing at 96ºC for 1 minute, annealing at 55ºC for 1 minute and extension at 72ºC for 1 minute, followed by a final extension at 72ºC for 10 minutes. Amplification products were purified and both strands sequenced using ABI 3100 and BigDye® chemistry (Applied Biosystems). Sequences were analysed and aligned using the Staden package (Staden *et al.*, 2000), BioEdit version 7.0.5.3, and ClustalW.

Table 8. Primers used to amplify the QRDR of topoisomerase genes

Primer	Nucleotide Sequence (5` – 3`)	Amplicon size (bp)	Reference
*gyrA*_fw	ATCTCGATTACGCGATGAGC	448	Pope *et al.* (2008)
*gyrA*_rv	GCCGTTGATCAGCAGGTT		
*gyrB*_fw	GAAGAAGTTGTGGCGAAGG	399	This study
*gyrB*_rv	AGTCTTCCTTGCCGATGC		Pope *et al.* (2008)
*parC*_fw	TACCTCAGCTACGCGGTCA	498	This study
*parC*_rv	GTTGTGCGACGGGATTTC		
*ParE*_fw	GAAGCTCGCCGAACTCGT	595	This study
*ParE*_rv	GTCCTTGCGCAGCTTGTC		

Footnotes:

Fw, forward primer; Rv, reverse primer.
bp, base pair.

2.14 BIOFILM ASSAYS

2.14.1 Biofilm biomass quantification in microtitre plates

An adapted biofilm assay (Peeters *et al.*, 2008b) was used to determine biofilm biomass production of *B .lata* strain 383 and derivatives in the presence and absence of sub-inhibitory preservative concentrations. *B. multivorans* (ATTC 17616), a prolific and consistent biofilm biomass producer, was included as a control. Preservative concentrations evaluated ranged from half the MIC for the test organism, to zero. *B. multivorans* was assayed with preservative concentrations ranging from: 0.00034% v/v for the isothiazolinone blend, 0.0097% v/v methylisothiazolinone, 0.004% w/v benzisothiazolinone and 0.027% v/v DMDM hydantoin. *B. lata* strain 383 and derivatives were all assayed at concentrations ranging from: 0.0000117% v/v for the isothiazolinone blend, 0.000485% v/v methylisothiazolinone, 0.001% w/v benzisothiazolinone and 0.00675% v/v DMDM hydantoin. Both *B. multivorans* and *B. lata* strains were evaluated with preservative concentrations ranging from: 0.075% w/v benzethonium chloride, 0.125% v/v phenoxyethanol and 0.05% w/v methyl paraben. Biofilm formation of both strains in BSM (CYG) at pH 5 containing sodium benzoate concentrations 0 – 0.1% w/v was also evaluated.

Bacterial cultures were grown for 18 hours in BSM (CYG) broth as previously described and diluted to approximately 1 x 10^6 CFU/ml in BSM (CYG) broth with or without preservative. Eight replicate wells of a flat-bottom polystyrene 96-well microtitre plate (Fisher Scientific) were inoculated with 200 µl of the suspension. Eight wells filled with sterile BSM (CYG) broth served as blanks. Plates were incubated statically at 30ºC for 72 hours. After incubation, planktonic growth was removed and the plates washed three times with PBS. The remaining biofilm was fixed with 99% methanol for 15 min, after which the supernatant was removed and the plates air-dried. The fixed biofilm was stained for 5 minutes with 0.1% v/v filtered crystal violet. 0.1% v/v crystal violet was found to be the optimal concentration for staining (Peeters *et al.*, 2008a).The excess stain was removed by washing with running water and the plates air-dried. The bound crystal violet stain was released by adding 33% v/v glacial acetic acid (Sigma), and the biofilm biomass enumerated by reading the optical density at 570 nm using an automated microtitre plate reader. Experiments were repeated with different starting cultures to obtain biological replicates.

2.15 SWARMING MOTILITY ASSAYS

2.15.1 Swarming motility in the presence of sub-inhibitory preservative concentrations

An adapted agar dilution assay was used to investigate swarming motility of Bcc strains in the presence of sub-inhibitory preservative concentrations as follows: molecular grade agarose (Bioline, UK) was dissolved in isosensitest broth (ISO; Oxoid, UK), at 2 g/L and autoclaved. After autoclaving the agar was cooled to 45°C and an appropriate volume of preservative stock solution added; 30 ml of the soft agar was poured into triple-vented petri dishes, and dried under laminar flow for 20 minutes. Semi-solid swarm plates were produced with the addition of preservative concentrations ranging from: 0 – 0.000749% v/v for the isothiazolinone blend, 0 – 0.00097% v/v methylisothiazolinone, 0 – 0.002% w/v benzisothiazolinone, 0.15% w/v benzethonium chloride, 0 – 0.0135% v/v DMDM hydantoin, 0 – 0.25% v/v phenoxyethanol and 0 – 0.1% w/v methyl paraben. *B. cenocepacia* J2315 (LMG 16656) and *B. multivorans* (ATCC 17616) were evaluated with higher concentrations of the following preservatives ranging from: 0 – 0.000674% v/v isothiazolinone blend, 0 – 0.00194% v/v methylisothiazolinone, 0 – 0.008% w/v benzisothiazolinone and 0 – 0.054% v/v DMDM hydantoin. *B. lata* strain 383, *B. multivorans* ATCC 17616 (a reference Bcc strain with good reproducible swarming motility) and *B. cenocepacia J2315* were cultured in ISO broth at 37ºC, or 30°C for *B. lata*, for 18 hours and diluted to OD 1±0.2 (600nm). Approximately 5 x 10^5 CFU were inoculated into the agar at the centre of triplicate soft agar plate and left to dry for 15 minutes. Plates were incubated at 30ºC or 37ºC depending on the test organism, for 24 - 48 hours and the diameter of the swarm measured (mm).

2.15.2 Swarming motility in the presence of sub-inhibitory concentrations of chlorhexidine

Previous studies by Dr. Helen Rose (2009) demonstrated sub-inhibitory concentrations of the biocide chlorhexidine (0.1 - 5 µg), in semi-solid swarm plates consisting of 0.3% w/v nutrient agar supplemented with 0.5% w/v glucose, inhibited/repressed swarming motility of the control strain *B. multivorans* (ATCC 17616). To validate the modifications to the swarming assay used in this study, semi-solid ISO swarm plates were prepared with sub-inhibitory concentrations of chlorhexidine ranging from 0 – 5 µg/ml. *B. lata* 383, *B. cenocepacia J2315* and *B. multivorans* were assayed as previously described.

2.15.3 Swarming motility of *B. lata* 383 and preservative adapted derivatives

The adapted swarming assay was used to compare swarming motility of the parental *B. lata* strain 383 and preservative adapted derivatives. Semi-solid swarm plates (without preservatives) were prepared as previously described. *B. lata* strain 383, adapted derivatives and *B. multivorans* ATCC 17616 were assayed as previously described. Experiments were repeated with different starting cultures to obtain biological replicates.

2.16 EFFLUX INHIBITION ASSAY

The effect of efflux activity on isothiazolinone preservative and fluoroquinolone susceptibility of *B. lata* strain *383* and derivative 383-CMIT were studied using MC-207,110 L-Phe-Arg-β-napthylamide (PAβN) (Sigma Aldrich,UK); a broad spectrum EPI shown to effectively inhibit clinically relevant efflux pumps in Gram negative bacteria, especially those associated with the extrusion of quinolone antibiotics (Pagès & Amaral, 2009; Pagès *et al*., 2005; Rajendran *et al.,* 2010). The MIC of PAβN was determined using modified broth microdilution assay. Appropriate amounts of an aqueous stock solution of PAβN (100 mg/ml) was added to BSM (CYG) broth to achieve a range of concentrations 0 – 2.048 mg/ml. *B. lata* strain *383* and derivative 383-CMIT were cultured for 18 hours as previously described, and diluted to and optical density of 1±0.2 (600 nm) in BSM (CYG) broth. Approximately 1 x 10^5 CFU of the test organism were added to the test and control media in triplicate wells of a 96-well microtitre plate (200 µl). Microplates were incubated with shaking (150 rpm) at 30ºC for 24 hours; the MIC was designated as the concentration of preservative at which there was no visible bacterial growth.

A checkerboard assay was used to evaluate the effect of sub-inhibitory concentrations of PAβN on the MIC of ciprofloxacin and the blend of isothiazolinone preservatives. Concentrations of PAβN ranging from 0 – 0.512 mg/ml were evaluated in combination with ciprofloxacin at 0 - 30 µg/ml and in combination with the isothiazolinone preservative blend at a range of 0 – 0.001498% v/v. Strains were cultured and diluted to an optical density of 1±0.2 (600 nm) as previously described. Approximately 1 x 10^5 CFU of the test organism was added to the test and control media in triplicate wells of a 96-well microtitre plate (200 µl). Microplates were incubated with shaking (150 rpm) at 30ºC for 24 hours; the MIC was designated as the concentration of ciprofloxacin or preservative (alone and in combination with PAβN), at which there was no visible bacterial growth.

The fractional inhibitory concentrations for combinations of antimicrobial and PAβN were calculated from the MIC as described in section 2.12.2.3.

3 PRESERVATIVE ACTIVITY AGAINST BCC BACTERIA

3.1 INTRODUCTION

Bcc bacteria are isolated from remarkably diverse ecological niches such as soils, waters, rhizospheres, plants, animals, hospital environments, industrial processes and infected humans (Mahenthiralingam *et al.*, 2008; Vial *et al.*, 2011). Distribution of Bcc species is heterogeneous and is dependent on the ecological niche (Vial *et al.*, 2011). The characterisation of Bcc species diversity in various ecological niches has not been equally investigated, the deficiency in data perhaps distorting the actual distribution of species in many environments (Vial *et al.*, 2011). Historically, research had focused extensively on clinical epidemiology and to a lesser extent the rhizosphere. Bcc species diversity in other environmental niches, including the environmental-industrial niche, has not been extensively studied. Characterising Bcc species distribution in the environmental-industrial niche is innately difficult. Manufacturers may wish to protect their public reputation and prevent sensitive information passing to competitors and hence deal with incidents of contamination internally. Publication of such instances of contamination is often not a priority for industry. In addition, industry may lack the research expertise to identify accurately bacterial contaminants, especially with regard to diverse and dynamic taxonomic groups such as the Bcc. Bcc encountered as contaminants are not routinely identified to the species and strain level by manufacturers. Even larger studies of product recalls and reports of microbial diversity in industrial processes often generically report Bcc contaminants as "*B.cepacia*" (Jimenez, 2007). To date, there has not been a systematic genetic typing study of the distribution of Bcc species encountered in industrial processes that takes recent taxonomic progress into account. Consequently, only limited information of species diversity within this niche can be gathered from characterised outbreaks of nosocomial Bcc infection resulting from the use of contaminated industrial product. Applying genotypic methods to the characterisation of Bcc isolates from environmental-industrial sources that reside within the Mahenthiralingam group culture collection at Cardiff University may expand the current knowledge of species diversity within this niche.

Preservatives are used extensively in clinical, agricultural, domiciliary and industrial environments to reduce spoilage and the risk of infection. In industry, preservatives are

incorporated (at regulated low levels) into raw materials and finished product to keep the process hygienically secure. An inadequate or compromised preservative system can lead to spoilage. This may result in economic loss for the manufacturer and, depending on the spoilage organism, may pose a risk to the consumer. Bcc bacteria are frequently identified Gram-negative contaminants of industrial processes (Jimenez, 2007), suggesting a high tolerance to biocides (e.g. disinfectants) and preservatives used by the industry to prevent microbial contamination.

The intrinsic resistance of the Bcc to biocides and antibiotics is well documented (Mahenthiralingam *et al.*, 2005), and is an aspect of their biology that undoubtedly contributes to their success as opportunistic pathogens and contaminants of industrial processes. Despite this, the relationship between Bcc species diversity and susceptibility is poorly understood, with many studies outdated by recent advances in Bcc taxonomy and a considerable bias of literature focused on antibiotic resistance. A recent study by Rose *et al.* (2009) addressed these issues and surveyed the susceptibility of Bcc bacteria to biocides commonly used as anti-infective agents. Susceptibility to chlorhexidine, CPC, triclosan, benzalkonium chloride and povidone, varied considerably across the complex. Interestingly, *B. cenocepacia,* a prevalent species in clinical infection, generally demonstrated higher tolerance to biocides and antibiotics. Although recognised as important bacterial contaminants of preserved industrial products, preservative resistance of Bcc bacteria and the relationship between susceptibility and taxonomy have not been extensively studied.

The complex nature of many industrial products such as pharmaceuticals, cosmetics and personal care product formulation, makes their preservation a challenge. Often, a single agent (at permitted levels) is ineffective at protecting such products from microbial contamination (Denyer *et al.*, 1985); many agents do not have a broad spectrum of antimicrobial activity against both bacteria and fungi (Orth *et al.,* 2006). Combinations of preservatives (preservative systems) are frequently used to protect raw materials used in industrial process, and the finished product during consumer use. Occasionally, combinations of preservative agents show synergy, their combined effects greater than would be expected from a basic additive effect of the combined agents (Denyer *et al.*, 1985). The combination of phenoxyethanol and paraben preservatives is a well known synergistic preservative combination, and has been exploited commercially (Chopra,

1996). Identifying synergistic activity or super-additive activity against contaminating microorganisms such as Bcc bacteria has obvious advantages, and is of significant benefit to the manufacturer, offering enhanced preservative activity at lower concentrations.

Phenotypic adaptation from planktonic to sessile cells is an important intrinsic mechanism of antimicrobial resistance with regards to industry. Biofilms are microbial communities of surface attached sessile cells embedded in a matrix of self-produced extracellular polymeric substances (Donlan & Costerton, 2002). Sessile cells within a biofilm often have increased resistance to antimicrobial agents due to the restricted permeation of agents into the biofilm, decreased growth rates, the expression of various resistance genes and/or the presence of persister cells (Peeters *et al.*, 2008b; Lewis, 2001; Mah & O'Toole, 2001). The incomplete penetration of the antimicrobial agent into the biofilm often prevents its eradication and buys time for at least some of the cells (perhaps many) to adapt to the challenge and actively respond to it by deploying protective stress responses (Szomolay *et al.*, 2005). Consequently, biofilms in industrial water systems or pipelines have the potential to act as a persistent reservoir of resistant organisms. The formation of biofilm can be a specific defence reaction to the presence of antimicrobial agents (Hoffman *et al.*, 2005). Sub-inhibitory concentrations of antibiotics have been shown to stimulate biofilm formation and exopolysaccharide production in Gram-negative and Gram-positive bacteria (Hoffman *et al.*, 2005). A recent study by Rose *et al.* (2009) (unpublished) demonstrated sub-inhibitory concentrations of chlorhexidine, and to a lesser extent CPC, biocides altered the motility of Bcc bacteria, inducing biofilm formation and inhibiting swarming motility. Sub-inhibitory concentrations of chlorhexidine did not elicit the same response in all Bcc strains, suggesting the response to be strain specific (Rose, 2009).In addition, other biocides such as triclosan and benzalkonium chloride, failed to elicit the same effect on Bcc motility and biofilm formation, indicating that the response may also be agent-specific. To protect the consumer, preservative agents are used at concentrations far lower than those of disinfectant or antiseptic agents. A compromised or inadequate preservative system in industrial processes may expose contaminating Bcc bacteria to sub-inhibitory concentrations. The transition from motility to sessility, and the induction of Bcc biofilm as a specific defence reaction in response to the presence of sub-inhibitory preservative agents, has not yet been investigated. The identification and avoidance of preservative agents that may elicit such a response in Bcc bacteria may be of significant benefit to manufacturers.

3.1.1 Aims

The aims of this chapter were as follows:

1) to investigate Bcc species diversity of a collection of isolates from environmental-industrial sources subjected to MLST analysis;

2) to investigate the preservative susceptibility of Bcc bacteria, exploring the relationship that Bcc species diversity and isolation source have with preservative susceptibility;

3) to conduct pairwise comparisons of isothiazolinone preservatives and other preservatives and preservative enhancers, in an attempt to identify combinations with high anti-Bcc activity;

4) to examine the effect of sub-inhibitory preservative concentrations on biofilm biomass production and swarming motility in Bcc bacteria.

3.2 RESULTS

3.2.1 Species diversity in a collection of Bcc isolates from environmental-industrial sources

MLST analysis of a collection of 67 Bcc isolates recovered from environmental-industrial sources revealed the following species diversity: *B. lata* (n = 17), *B. cenocepacia* (n = 11), Novel Bcc (n = 9), *B. vietnamiensis* (n = 8), *B. arboris* (n = 7), *B. stabilis* (n = 6), *B. cepacia* (n = 3), *B. multivorans* (n = 3), *B. contaminans* (n = 2), and *B. ambifaria* (n = 1). 57 of the isolates were assigned a sequence type (ST). Ten isolates were accurately identified to the species level by partial MLST analysis (i.e. fewer than seven loci were sequenced) (Table 9). A total of 33 ST were isolated from environmental-industrial sources reflecting the presence of duplicate strains within the collection. *B. lata*, the predominant species group in the collection, had a particularly low diversity of only 5 sequence types. A selection of 19 of these industrial isolates spanning 8 Bcc species groups, were included in a strain panel used in the evaluation of Bcc preservative susceptibility (sections 3.2.3 and 3.2.4; Table 6 for panel).

3.2.2 Bacterial growth on modified basal salts medium used in preservative susceptibility testing

A basal salts medium (Hareland *et al.*, 1975) was selected for use in the evaluation of Bcc preservative susceptibility. The modified minimal medium better represented environmental-industrial conditions and was found to be less detrimental to the recovery of stressed organisms in comparison to a nutrient rich medium (data not shown). The medium was supplemented with 0.5 g/L casamino acids and 0.5 g/L yeast extract to enable the evaluation of auxotrophic organisms. The medium was further modified by the removal of the chelating agent nitrilotriacetic acid, to avoid the potentiation of preservative activity. Growth of nine Bcc strains in modified and unmodified BSM (CYG) broth was evaluated as described in section 2.4.4. A Mann-Whitney (two-tailed) statistic test confirmed that there was no significant difference ($P > 0.05$) in the number of viable cells of modified and unmodified medium at 24 hours ; strains consistently reached between 1×10^8 CFU/ml and 1×10^9 CFU/ml in modified and unmodified medium. The removal of the chelating agent did not result in the precipitation of metal salts or prove detrimental in turbidimetric assay.

Table 9. Bcc species diversity in a collection of environmental-industrial isolates revealed by MLST analysis

Species/Group	Strain ID	ST	Source
B. ambifaria	BCC1401	454	Process contaminant
B. arboris	BCC1306	325	Process contaminant
B. arboris	BCC1307	325	Process contaminant
B. arboris	BCC1326	325	Process contaminant
B. arboris	BCC1310	327	Process contaminant
B. arboris	BCC1312	328	Process contaminant
B. arboris	VAN01	349	Pharmaceutical product
B. arboris	BCC1395	-¥	Process contaminant
B. cenocepacia IIIA	BCC1295	241	Process contaminant
B. cenocepacia IIIA	BCC1283	250	Process contaminant
B. cenocepacia IIIA	BCC1558	-¥	Process contaminant
B. cenocepacia IIIB	BCC1318	316	Process contaminant
B. cenocepacia IIIB	BCC1320	316	Process contaminant
B. cenocepacia IIIB	BCC1324	316	Process contaminant
B. cenocepacia IIIB	BCC1291	322	Process contaminant
B. cenocepacia IIIB	BCC1292	322	Process contaminant
B. cenocepacia IIIB	BCC1293	322	Process contaminant
B. cenocepacia IIIB	BCC1316	338	Process contaminant
B. cenocepacia IIIB	BCC1322	340	Process contaminant
B. cepacia	BCC1407	3	Process contaminant
B. cepacia	BCC1404	205	Process contaminant
B. cepacia	BCC1305	324	Process contaminant
B. contaminans	BCC1323	323	Process contaminant
B. contaminans	BCC1315	341	Process contaminant
B. lata	BCC1284	98	Process contaminant
B. lata	BCC1285	98	Process contaminant
B. lata	BCC1286	98	Process contaminant
B. lata	BCC1287	98	Process contaminant
B. lata	BCC1288	98	Process contaminant
B. lata	BCC1289	98	Process contaminant
B. lata	BCC1290	98	Process contaminant
B. lata	BCC1294	98	Process contaminant
B. lata	BCC1406	103	Process contaminant
B. lata	BCC1296	119	Process contaminant
B. lata	BCC1297	119	Process contaminant
B. lata	BCC1298	119	Process contaminant
B. lata	BCC1299	119	Process contaminant
B. lata	BCC1554	119	Process contaminant
B. lata	BCC1321	339	Process contaminant
B. lata	VAN 05	342	Process contaminant
B. lata	VAN 06	-¥	Process contaminant

Table 9. Bcc species diversity in a collection of environmental-industrial isolates revealed by MLST analysis (continued)

Species/Group	Strain ID	ST	Source
Bcc group Kc	VAN 03	333	Process contaminant
Bcc group Kc	VAN 04	333	Process contaminant
Bcc group Kc	BCC1282	333	Process contaminant
Bcc group Kc	BCC1302	333	Process contaminant
Bcc group Kc	BCC1303	333	Process contaminant
Bcc group Kc	BCC1300	334	Process contaminant
Bcc group Kc	BCC1313	335	Process contaminant
Bcc group Kc	BCC1314	336	Process contaminant
Bcc group Kc	BCC1410	455	Aircraft diesel container
B. multivorans	VAN 02	373	Process contaminant
B. multivorans	BCC1559	439	Process contaminant
B. multivorans	BCC1560	439	Process contaminant
B. stabilis	BCC1207	50	Pharmaceutical product
B. stabilis	VAN 07	50	Pharmaceutical product
B. stabilis	BCC0479	51	Process contaminant
B. stabilis	BCC1308	337	Process contaminant
B. stabilis	BCC1325	-¥	Process contaminant
B. stabilis	BCC1555	-¥	Process contaminant
B. vietnamiensis	BCC0195	60	Process contaminant
B. vietnamiensis	BCC1301	60	Process contaminant
B. vietnamiensis	BCC1309	326	Process contaminant
B. vietnamiensis	BCC1304	-¥	Process contaminant
B. vietnamiensis	BCC1408	-¥	Aircraft diesel container
B. vietnamiensis	BCC1409	-¥	Aircraft diesel container
B. vietnamiensis	BCC1411	-¥	Aircraft diesel container
B. vietnamiensis	BCC1412	-¥	Aircraft diesel container

Footnotes:

ST, sequence type of isolate

¥ Isolate identified to species level only, not assigned a sequence type

3.2.3 Preservative susceptibility of a collection of eight Bcc bacteria

Prior to the evaluation of a collection of 83 Bcc strains spanning the genetically diverse 17 species groups, the susceptibility of a panel of eight Bcc strains (Table 5) to nine preservatives and three preservative enhancing agents (Table 10) was determined by broth or agar dilution assays. The preliminary strain collection represented species commonly encountered in a collection of industrial isolates, and included five strains originally isolated from industrial sources. Due to the limited solubility of propyl paraben in aqueous solution (0.4 g/L), the susceptibility of Bcc bacteria could not be determined using broth or agar dilution methods. Approximate MIC values for *B. cenocepacia* J2315 and *B. lata* strain 383 determined by a broth dilution assay, indicated that minimum inhibitory concentrations exceeded those of the upper limit of solubility; consequently, this agent was not evaluated further. Solubility issues surrounding the preservative enhancing agents caprylyl glycol and Ethylhexyl glycerin also hindered susceptibility testing. In broth dilution assays, higher concentrations of the preservative enhancers were opaque or formed precipitate, preventing the measurement of bacterial growth by turbidometric analysis. Higher concentrations of the aforementioned preservative enhancers formed a hydrophobic layer on the surface of BSM (CYG) agar, preventing plating of bacteria.

Preservative susceptibility varied between and within species groups. None of the eight strains evaluated had a broad-ranging high tolerance to all nine preservatives evaluated. For example, *B. lata* strain Bcc1294 had among the highest MIC values for isothiazolinone, DMDM hydantoin and sodium benzoate preservatives, but among the lowest MIC values to phenoxyethanol and methyl paraben. MIC values for isothiazolinone, phenoxyethanol, DMDM hydantoin and paraben preservatives were below the maximum levels permitted for use in personal care products in EU-regulated countries (Table 2). However, *B. cenocepacia* HI2424 had an MIC greater than the permitted levels for benzethonium chloride (>0.1% w/v). All eight strains had MICs greater than or equal to the maximum permitted level of sodium benzoate (≥0.5% w/v). However, preservative susceptibility testing of this agent was initially performed in medium with a neutral pH (pH 7) and this may have reduced preservative activity which is optimal at pH 5. *B.cenocepacia* J2315, a multidrug-resistant clinical strain with increased tolerance to biocides (Rose *et al.*, 2009), had the lowest MIC values of the collection for four of the nine preservatives evaluated. All Bcc strains had MIC values greater than the maximum permitted level for the preservative enhancer EDTA (>0.1% v/v).

3.2.3.1 Preservative susceptibility of three non-Bcc reference strains

E.coli (NTCC 1241), *S. aureus* (NTCC 12981) and *P. aeruginosa* (NTCC 12903) were evaluated with the highest and lowest MIC values of the panel of eight Bcc bacteria using a broth dilution method. The minimum inhibitory concentrations of the non-Bcc species in relation to the collection of Bcc bacteria are shown in Table 11. The preliminary evaluation of susceptibility indicated that Bcc bacteria were more susceptible to phenoxyethanol, methyl paraben and sodium benzoate, but more tolerant to benzethonium chloride and EDTA.

Table 10. The susceptibility of eight Bcc strains to preservatives and preservative enhancing agents

Species	MIC (% [2]) values for preservative and preservative enhancers											
Strain designation	MIT	MIT/CMIT[1]	BIT	PH	BC	DMH	SB	MP	PP	EDTA	CG	EG
B.arboris												
Bcc1306	0.0007	0.0000375	0.001000	0.15	<0.0075	0.054	0.50	0.075	N/A	2	N/A	>2
B. cenocepacia												
J2315 [T]	0.0005	<0.0000094	0.00004	0.15	0.015	0.0027	0.75	0.075	>0.04	2	>0.25	0.5
HI2424	0.0010	0.0000749	0.0011	0.15	>0.1	0.00405	0.5	0.075	N/A	2	N/A	>2
Bcc1283	0.0007	0.0005243	0.0011	0.025	0.05	0.0405	0.5	0.075	N/A	2	N/A	0.5
Bcc 1291	0.0010	0.0000749	0.0013	0.15	0.01	0.054	0.5	0.075	N/A	2	N/A	1
B.lata												
Strain 383 [T]	0.0010	0.0000375	0.002000	0.15	0.0100	0.00405	0.75	0.075	>0.04	3	>0.25	1
Bcc1294	0.0010	0.0004494	>0.0016	0.05	0.0500	0.0405	0.75	0.050	N/A	3	N/A	2
Bcc1296	0.0007	0.0001124	>0.002	0.15	0.0150	0.0041	0.75	0.075	N/A	4	N/A	1

Footnotes:

MIC, minimum inhibitory concentration. MIT, methylisothiazolinone; MIT/CMIT, methylisothiazolinone and chloromethylisothiazolinone; BIT, benzisothiazolinone; PH, phenoxyethanol; BC, benzethonium chloride; DMH, dimethylol dimethyl hydantoin; SB, sodium benzoate; MP, methyl paraben; PP, propyl paraben; EDTA, Ethylenediaminetetraacetic acid; CG, caprylyl glycol; EG, Ethylhexyl glycol.

[1] A cosmetic grade commercial blend evaluated

[2] % refers to v/v for MIT, MIT/CMIT, PH, DMH and EDTA ; % refers to w/v for other agents.

[T] Type strain

N/A, not assayed

Table 11. Susceptibility of three non-Bcc reference strains to eight preservatives and EDTA

Species	MIC (%) values for preservative and preservative enhancers								
Strain designation	MIT	MIT/CMIT[1]	BIT	PH	BC	DMH	SB	MP	EDTA
Escherichia coli									
NTCC 1241	0.00097	0.0000749	<0.001	>0.15	<0.005	0.027	0.50	>0.075	<0.5
Staphylococcus aureus									
NTCC 12981	0.00097	0.0000749	<0.001	>0.15	<0.005	0.027	>0.75	>0.075	<0.5
Pseudomonas aeruginosa									
NTCC 12903	0.00097	0.0005243	>0.002	>0.15	<0.005	0.027	>0.75	>0.075	<0.5
Highest Bcc MIC value [2]	0.00097	0.0005243	0.002	0.15	>0.1	0.054	0.75	0.075	4

Footnotes:

MIC, minimum inhibitory concentration. MIT, methylisothiazolinone; MIT/CMIT, methylisothiazolinone and chloromethylisothiazolinone; BIT, benzisothiazolinone; PH, phenoxyethanol; BC, benzethonium chloride; DMH, dimethylol dimethyl hydantoin; SB, sodium benzoate; MP, methyl paraben; EDTA, Ethylenediaminetetraacetic acid.

[1] A cosmetic grade commercial blend evaluated

[2] The highest MIC value for a panel of eight Bcc bacteria.

% refers to v/v for MIT, MIT/CMIT, PH, DMH and EDTA ; % refers to w/v for other agents.

Due to solubility issues propyl paraben, caprylyl glycol and Ethylhexyl glycerin were not assayed.

3.2.4 Preservatives susceptibility of a collection of 83 Bcc bacteria

3.2.4.1 Minimum inhibitory concentrations

An agar dilution assay was used to determine the minimum inhibitory concentrations of a collection of 83 Bcc bacteria and ten non-Bcc bacteria for seven preservatives used in industrial processes. Preservative concentrations evaluated were based on the preliminary assay of a collection of eight Bcc strains and three non-Bcc reference strains. The mean and range MIC values (Table 12) demonstrated that preservative susceptibility varied considerably both between and within species of the Bcc: for example, the MIC values for the blend of isothiazolinone preservatives were widely distributed (Figure 9, panel A). The majority of Bcc strains (≥50 out of 83) shared a common MIC value for benzethonium chloride, DMDM hydantoin, phenoxyethanol and methyl paraben preservatives: 0.15% w/v, 0.0135% v/v, 0.25% v/v and 0.1% w/v respectively (Table 12). MIC values for isothiazolinone preservatives and sodium benzoate were perhaps better resolved by the range of concentrations evaluated (increasing two-fold), with the MIC values for ≥28 strains clustering at two concentrations. For example, 34 and 31 (out of 83) Bcc strains had MIC values of 0.0000749% v/v and 0.00015% v/v respectively, for the isothiazolinone blend (Figure 9). The identification of species-dependent differences in preservative susceptibility was not obvious, due to the considerable variation within species and MLST groups. Statistical analysis of many species or MLST groups was not performed, as 12 of the (21) groups evaluated had few (≤ 3) representative strains within the collection. None of the species groups, including *B. lata* (the predominating species in a collection of Bcc isolated from environmental-industrial sources), demonstrated multi-preservative resistance (MPR).

Susceptibility profiling revealed Bcc strains with high MIC values (≥ the highest concentration evaluated) for related and unrelated preservatives with different cellular targets. For example, *B.cenocepacia* strain Bcc1203 had high MIC values for the blend of isothiazolinone preservatives (0.00064% v/v), benzisothiazolinone (0.008% w/v), and the unrelated benzethonium chloride (0.4% w/v); they also displayed mid-range tolerance to phenoxyethanol (0.25% v/v) and sodium benzoate (0.4% w/v) preservatives. However, a high MIC value did not necessarily predict higher MIC values for preservatives of the same class; *B. cenocepacia* Bcc1291 had high MIC values for methylisothiazolinone (0.0004365% v/v) and the isothiazolinone preservative blend (0.00015% v/v), but a lower MIC for benzisothiazolinone (0.001% w/v). Preservative susceptibility (by MIC) also varied between

isolates with identical sequence types (MLST analysis). *B.cenocepacia* H12424, an environmental isolate of ST-122, had higher MIC values for the blend of isothiazolinone (0.000674% v/v), benzisothiazolinone (0.008% w/v) and benzethonium chloride (0.4% w/v) preservatives than the clinical isolate *B.cenocepacia* AU1054 (ST-122). *B. dolosa* strains Bcc0072 and AU0645, both clinical isolates of ST-72, had identical MIC values for all preservatives except methyl paraben and benzisothiazolinone.

3.2.4.2 Minimum bactericidal concentrations

The MBC of six preservatives for the collection of 83 Bcc strains and 10 non-Bcc bacterial species was determined using a modified broth dilution assay as described in section 2.12.4.2. Methyl paraben was not evaluated, as bactericidal concentrations (>0.2% w/v) could not be achieved in aqueous solution due to a limited solubility. The efficacy and toxicity of the neutralising solution (containing 2% w/v Tween80 and 0.1% w/v peptone) was evaluated prior to experimentation, as described in section2.12.4.1. To assess toxicity, approximately 10^5 CFU of *B. lata* strain 383 were exposed to the neutraliser or water as a control. A (Two-tailed) Mann-Whitney statistical test confirmed that there was no significant difference ($P > 0.05$) in the number of viable cells recovered after exposure to the neutralising solution. To assess the efficacy of terminating activity of the preservatives, approximately 10^5 CFU of *B. lata* strain 383 were exposed to putatively lethal concentrations of preservatives (higher than the MIC) that had been neutralised in a solution of 2% w/v Tween 80 and 0.1% w/v peptone solution, or in deionised water as a control. Approximately 10^5 CFU/ml were recovered after exposure to preservatives neutralised by the 2% w/v Tween 80 and 0.1% w/v peptone solution. The enumeration of viable cells indicated that the inactivation of preservatives by the neutralising solution may not have been 100% effective; growth of viable cells was slightly inhibited at lower dilutions in the dilution series. To improve the efficacy of preservative inactivation and reduce the concentration to less than the MIC, an additional 1 in 10 dilution into an “enrichment plate”, followed by an 18 hour culture, was performed for higher test concentrations.

Table 12. Mean and range MIC values of preservatives for Bcc and other bacterial species tested

Species/group	MIC (%) values for preservatives															
	MIT		MIT/CMIT[1]		BIT		BC		DMH		MP		PH		SB	
(number of strains tested)	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
B. ambifaria (4)	0.00146	0.00097 - 0.00194	0.000084	0.000037 - 0.000150	0.0015	0.0010 - 0.0020	0.114	0.005 - 0.150	0.0135	[a]	0.088	0.050 - 0.100	0.219	0.125 - 0.250	0.250	0.200 - 0.400
B. anthina (2)	0.00194	[a]	0.000112	0.000075 - 0.000150	0.0030	0.0020 - 0.0040	0.150	[a]	0.0135	[a]	0.100	[a]	0.250	[a]	0.400	[a]
B. arboris (4)	0.00121	0.00097 - 0.00194	0.000066	0.000037 - 0.000075	0.0020	0.0010 - 0.0040	0.150	[a]	0.0911	0.0135 - 0.2160	0.088	0.050 - 0.100	0.144	0.125 - 0.150	0.175	0.100 - 0.200
B. cenocepacia (15)	0.00158	0.00049 - 0.00437	0.000170	0.000037 - 0.000674	0.0023	0.0010 - 0.0080	0.201	0.010 - 0.400	0.0450	0.0135 - 0.2160	0.080	0.050 - 0.100	0.179	0.125 - 0.250	0.280	0.100 - 0.800
B. cepacia (4)	0.00133	0.00049 - 0.00194	0.000094	0.000037 - 0.000150	0.0030	0.0010 - 0.0080	0.114	0.008 - 0.150	0.0135	[a]	0.100	[a]	0.200	0.150 - 0.250	0.375	0.100 - 0.800
B. contaminans (5)	0.00155	0.00097 - 0.00194	0.000120	0.000075 - 0.000150	0.0016	0.0010 - 0.0020	0.20 [1]	0.150 - >0.400	0.0594	0.0135 - 0.1080	0.100	[a]	0.250	[a]	0.280	0.200 - 0.400
B. diffusa (3)	0.00162	0.00097 - 0.00194	0.000100	0.000075 - 0.000150	0.0013	0.0010 - 0.0020	0.150	[a]	0.0135	[a]	0.100	[a]	0.250	[a]	0.200	[a]
B. dolosa (3)	0.00162	0.00097 - 0.00194	0.000125	0.000075 - 0.000150	0.0013	0.0010 - 0.0020	0.150	[a]	0.0135	[a]	0.067	0.050 - 0.100	0.125	[a]	0.100	[a]
B. lata (5)	0.00097	[a]	0.000210	0.000075 - 0.000674	0.0026	0.0010 - 0.0040	0.121	0.005 - 0.150	0.0486	0.0135 - 0.1080	0.100	[a]	0.225	0.125 - 0.250	0.280	0.200 - 0.400
B. metallica (2)	0.00146	0.00097 - 0.00194	0.000112	0.000075 - 0.000150	0.0020	[a]	0.150	[a]	0.0135	[a]	0.100	[a]	0.250	[a]	0.300	0.200 - 0.400
B. multivorans (8)	0.00158	0.00049 - 0.00437	0.000145	0.000037 - 0.000674	0.0024	0.0010 - 0.0080	0.150	[a]	0.0439	0.0135 - 0.2160	0.094	0.050 - 0.100	0.169	0.125 - 0.250	0.238	0.100 - 0.400
B. pyrrocinia (3)	0.00129	0.00097 - 0.00194	0.000075	[a]	0.0010	[a]	0.150	[a]	0.0135	[a]	0.100	[a]	0.250	[a]	0.167	0.100 - 0.200
B. stabilis (3)	0.00146	0.00049 - 0.00194	0.000112	0.000037 - 0.000150	0.0017	0.0010 - 0.0020	0.150	[a]	0.0135	[a]	0.100	[a]	0.208	0.125 - 0.250	0.233	0.100 - 0.400
B. ubonensis (1)	0.00097	[¥]	0.000150	[¥]	0.0020		0.150	[¥]	0.0135	[¥]	0.100	[¥]	0.250	[¥]	0.100	[b]
B. vietnamiensis (5)	0.00116	0.00097 - 0.00194	0.000127	0.000037 - 0.000150	0.0024	0.0020 - 0.0040	0.038	0.008 - 0.150	0.0351	0.0135 - 0.1080	0.090	0.050 - 0.100	0.165	0.125 - 0.250	0.420	0.100 - 0.800
BCC Kc (4)	0.00133	0.00049 - 0.00194	0.000243	0.000075 - 0.000674	0.0018	0.0010 - 0.0040	0.114	0.005 - 0.150	0.0945	0.0540 - 0.1080	0.125	0.100 - 0.200	0.250	[a]	0.400	[a]
BCC4 (3)	0.00129	0.00097 - 0.00194	0.000100	0.000075 - 0.000150	0.0023	0.0010 - 0.0040	0.150	[a]	0.0180	0.0135 - 0.0270	0.100	[a]	0.250	[a]	0.167	0.100 - 0.200
BCC5 (3)	0.00129	0.00097 - 0.00194	0.000075	[a]	0.0013	0.0010 - 0.0020	0.150	[a]	0.0135	[a]	0.083	0.050 - 0.100	0.208	0.125 - 0.250	0.167	0.100 - 0.200
BCC6 (3)	0.00194	[a]	0.000150	[a]	0.0027	0.0020 - 0.0040	0.150	[a]	0.0135	[a]	0.100	[a]	0.250	[a]	0.333	0.200 - 0.400
BCC8 (1)	0.00194	[¥]	0.000075	[¥]	0.0020	[¥]	0.150	[¥]	0.1080	[¥]	0.100	[¥]	0.250	[¥]	0.200	[b]
unclassified BCC (2)	0.00146	0.00097 - 0.00194	0.000112	0.000075 - 0.000150	0.0020	[a]	0.150	[a]	0.0135	[a]	0.100	[a]	0.250	[a]	0.300	0.200 - 0.400
non Bcc (10)	0.00141	0.00097 - 0.00437	0.000075	0.000037 - 0.000150	0.0015	0.0010 - 0.0040	0.049	0.005 - 0.150	0.0243	0.0135 - 0.1080	0.105	0.050 - 0.400	0.233 [1]	0.125 - >0.300	0.130	0.100 - 0.400

Footnotes:

MIC, minimum inhibitory concentration. MIT, methylisothiazolinone; MIT/CMIT, methylisothiazolinone and chloromethylisothiazolinone; BIT, benzisothiazolinone; PH, phenoxyethanol; BC, benzethonium chloride; DMH, dimethylol dimethyl hydantoin; SB, sodium benzoate; MP, methyl paraben.

[1] A cosmetic grade commercial blend evaluated.

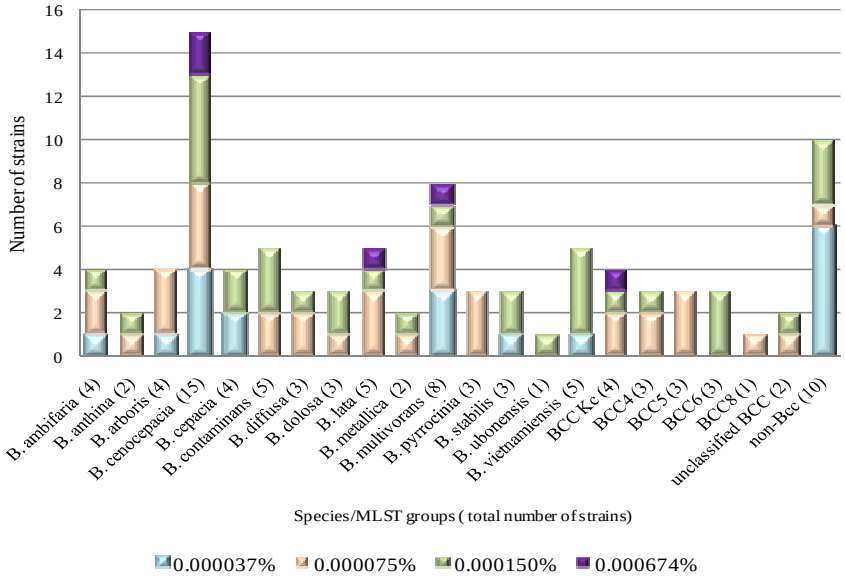
[X] Number of strains with an MIC greater than the highest concentration evaluated.

[a] Values the same for all strains within group .

¥ Only 1 strain evaluated in group.

% refers to v/v for MIT, MIT/CMIT, PH and DMH; % refers to w/v for other agents.

A) MIC variation across the *B. cepacia* complex



B) MBC variation across the *B. cepacia* complex

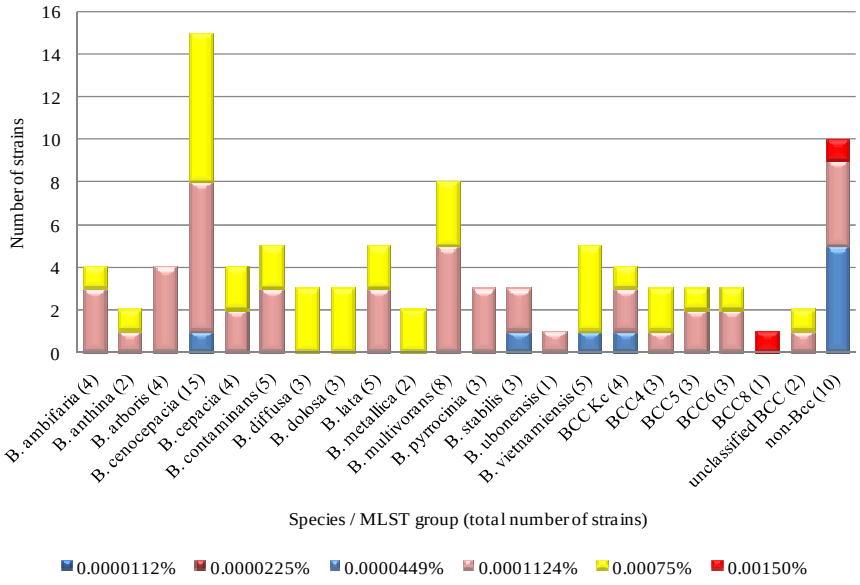


Figure 9. The susceptibility of Bcc and other bacterial species to the cosmetic grade blend of isothiazolinone preservatives.

Panels A and B show the distribution of MIC and MBC values (% v/v) respectively of the isothiazolinone preservative blend for Bcc and other bacterial species tested. The legend shows preservative concentration (%). The maximum level for use in rinse-off personal care products in EU regulated countries is 0.0015% (EU cosmetics directive 76/768/EEC Annex VI).

The mean and range MBC values (Table 13) demonstrated that none of the species groups had broad-ranging resistance to killing as the preservative susceptibility varied considerably both between and within species of the Bcc. As with MIC susceptibility testing, the considerable variation of MBCs within species/MLST groups, and the limited number of strains evaluated for some species groups, hindered the identification and statistical analysis of species-specific differences. Examples of the distribution of MBC values within and between species groups for the blend of isothiazolinone preservatives are shown in Figure 9. As observed with MIC values, the majority of Bcc strains (≥60 out of 83) shared common MBCs for benzethonium chloride, DMDM hydantoin and phenoxyethanol: 0.05% w/v, 0.081% v/v, and 0.5% v/v respectively. MBC values for isothiazolinone and sodium benzoate preservatives clustered at two values. For example, 42 and 36 (out of 83) Bcc strains had MBC values of 0.0001124% v/v and 0.00075% v/v for the isothiazolinone blend respectively. Four strains (*B.cenocepacia, B. stabilis, B. vietnamiensis,* BCC Kc) were more susceptible, with a lower MIC value of 0.0000449% v/v. One strain (BCC8) was more tolerant, with a higher MIC of 0.0015% v/v (Figure 9).

The highest evaluated concentrations of DMDM hydantoin (0.081% v/v) and phenoxyethanol (5% v/v) were bactericidal for all 83 Bcc strains evaluated; the highest MBC values were 1.5 and four-fold greater than the highest MIC values for the respective preservatives. The highest evaluated concentrations of methylisothiazolinone (0.01% v/v), the isothiazolinone blend (0.0015% v/v), benzisothiazolinone (0.015% w/v) and sodium benzoate (2.5% w/v), were sufficient to kill 82 out of 83 strains. Within each case, a different isolate was resistant to killing: *B. dolosa* strain 1356 survived killing with MIT; BCC8 (Bcc1191) survived killing with MIT/CMIT; *B. diffusa* strain Bcc0169 survived killing with BIT; and *B. multivorans* strain Bcc0005 survived killing with sodium benzoate. Eleven Bcc strains survived exposure to the highest evaluated concentration of benzethonium chloride (0.1% w/v), comprising nine *B. cenocepacia* strains, *B. contaminans* strain Bcc1323 and BCC Kc strain Bcc1313. Overall, MBC values for isothiazolinone, benzethonium and sodium benzoate preservatives were greater than two-fold that of the highest MIC values.

Table 13. Mean and range MBC values of preservatives for Bcc and other bacterial species tested

Species/group (number of strains tested)	MBC (%) values for preservatives													
	MIT		MIT/CMIT [1]		BIT		BC		DMH		PH		SB	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
B. ambifaria (4)	0.00170	0.00097 - 0.00194	0.0002718	0.000112 - 0.000750	0.0019	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.500	[a]	0.750	0.500 - 1.000
B. anthina (2)	0.00340	0.00194 - 0.00485	0.0004312	0.000112 - 0.000750	0.0023	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.750	0.500 - 1.000	1.750	1.000 - 2.500
B. arboris (4)	0.00267	0.00194 - 0.00485	0.000112	[a]	0.0026	0.0015 - 0.0060	0.050	[a]	0.162	0.081 - 0.324	0.500	[a]	1.250	0.500 - 2.500
B. cenocepacia (15)	0.00407	0.00194 - 0.00485	0.0004554	0.000045 - 0.001500	0.0028	0.0015 - 0.0060	0.63 [9]	0.050 - >1.000	0.097	0.081 - 0.162	0.633	0.500 - 1.000	2.050	0.250 - 2.500
B. cepacia (4)	0.00267	0.00194 - 0.00485	0.0004312	0.000112 - 0.000750	0.0034	0.0015 - 0.0060	0.050	[a]	0.081	[a]	0.500	[a]	2.000	0.500 - 2.500
B. contaminans (5)	0.00369	0.00194 - 0.00485	0.0003674	0.000112 - 0.000750	0.0021	0.0015 - 0.0030	0.24 [1]	0.050 - >1.000	0.113	0.081 - 0.162	0.600	0.500 - 1.000	1.600	1.000 - 2.500
B. diffusa (3)	0.00388	0.00194 - 0.00485	0.0007500	0.000750 - 0.000750	0.007 [1]	0.0030 - >0.0150	0.050	[a]	0.081	[a]	0.667	0.500 - 1.000	2.000	1.000 - 2.500
B. dolosa (3)	0.0100 [1]	0.01000 - >0.01000	0.0007500	0.000750 - 0.000750	0.0050	0.0030 - 0.0060	0.050	[a]	0.081	[a]	0.500	[a]	2.500	[a]
B. lata (5)	0.00230	0.00097 - 0.00485	0.0003674	0.000112 - 0.000750	0.0054	0.0015 - 0.0150	0.060	0.050 - 0.100	0.113	0.081 - 0.162	0.500	[a]	1.300	1.000 - 2.500
B. metallica (2)	0.00340	0.00194 - 0.00485	0.0007500	0.000750 - 0.000750	0.0023	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.500	[a]	2.500	[a]
B. multivorans (8)	0.00456	0.00097 - 0.01000	0.0003515	0.000112 - 0.000750	0.0026	0.0015 - 0.0030	0.050	[a]	0.111	0.081 - 0.324	0.625	0.500 - 1.000	2.5 [1]	2.500 - >2.500
B. pyrrocinia (3)	0.00194	[a]	0.000112	[a]	0.0020	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.500	[a]	1.500	1.000 - 2.500
B. stabilis (3)	0.00162	0.00097 - 0.00194	0.000090	0.000045 - 0.000112	0.0025	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.500	[a]	1.833	0.500 - 2.500
B. ubonensis (1)	0.00194	¥	0.000112	¥	0.0030	¥	0.050	¥	0.081	¥	0.050	¥	0.050	¥
B. vietnamiensis (5)	0.00427	0.00194 - 0.00485	0.0006090	0.000045 - 0.000750	0.0024	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.800	0.500 - 1.000	1.450	[a]
BCC Kc (4)	0.00170	0.00097 - 0.00194	0.0002549	0.000045 - 0.000750	0.0026	0.0015 - 0.0030	0.325 [1]	0.050 - >1.000	0.122	0.081 - 0.162	0.500	[a]	1.375	1.000 - 2.500
BCC4 (3)	0.00388	0.00194 - 0.00485	0.0005375	0.000112 - 0.000750	0.0035	0.0015 - 0.0060	0.050	0.050 - 0.050	0.081	[a]	0.833	0.500 - 1.000	2.500	[a]
BCC5 (3)	0.00291	0.00194 - 0.00485	0.0003249	0.000112 - 0.000750	0.0020	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.500	[a]	1.000	[a]
BCC6 (3)	0.00291	0.00194 - 0.00485	0.0003249	0.000112 - 0.000750	0.0030	[a]	0.100	0.050 - 0.200	0.081	[a]	0.500	[a]	2.000	1.000 - 2.500
BCC8 (1)	0.00485	¥	>0.0015 [1]	¥	0.0015	¥	0.050	¥	0.081	¥	0.050	¥	0.050	¥
unclassified BCC (2)	0.00340	0.00194 - 0.00485	0.0004312	0.000112 - 0.000750	0.0023	0.0015 - 0.0030	0.050	[a]	0.081	[a]	0.500	[a]	2.500	[a]
non Bcc (10)	0.00375	0.00097 - 0.01000	0.0002812 [1]	0.000045 - >0.001500	0.00345	0.0015 - >0.0150	0.109	0.050 - 1.000	0.089	0.081 - 0.162	1.125	0.500 - 5.000	1.484 [1]	0.250 - >2.500

Footnotes:

MBC, minimum bactericidal concentration. MIT, methylisothiazolinone; MIT/CMIT, methylisothiazolinone and chloromethylisothiazolinone; BIT, benzisothiazolinone; PH, phenoxyethanol; BC, benzethonium chloride; DMH, dimethylol dimethyl hydantoin; SB, sodium benzoate.

[1] A cosmetic grade commercial blend evaluated.

[X] Number of strains with an MBC greater than the highest concentration evaluated.

[a] Values the same for all strains within group.

¥ Only 1 strain evaluated in group.

Preservative susceptibility of Bcc strains varied considerably by MBC, within species groups. Susceptibility profiling identified Bcc strains with high MBC values (≥ the highest concentration evaluated) for related and unrelated preservatives with different cellular targets. Preservative susceptibility profiles (MBC values) of select strains, including the recommended preservative efficacy testing challenge test organism *B. cepacia* LMG 1222 (ATCC 25416), with high MBC values for multiple preservatives or DMDM hydantoin, are shown in Table 15. Preservative susceptibility of Bcc strains of the same sequence type varied less by MBC: *B. dolosa* strains Bcc0072 and Bcc0305 (ST−72) had identical MBC values for all preservatives except benzisothiazolinone (MBCs 0.006% and 0.003% respectively). *B. cenocepacia* AU1054 and H1244, both ST−122, had identical MBC values for all preservatives evaluated.

3.2.4.3 Bcc preservative susceptibility in relation to isolation source

The relationship between the original isolation source and Bcc preservative susceptibility was investigated. The collection of 83 Bcc strains used for preservative susceptibility testing were originally from clinical (n = 41), environmental (n = 24) and environmental-industrial (n =18) sources. Species and number of strains from each isolation source are shown in Table 14. Significant differences in the minimum inhibitory and bactericidal concentrations of isolates from different sources were determined using a Kruskal-Wallis and Mann-Whitney (Two-tailed) statistical tests. Bacteria isolated from environmental-industrial sources had significantly higher ($P < 0.0001$) mean MIC and MBC values for DMDM hydantoin than Bcc from clinical or environmental sources (Figure 10). Mean MBC values of other preservatives did not significantly differ ($P > 0.05$) in relation to the source of Bcc isolation. The mean MICs of phenoxyethanol (0.25%) and methyl paraben (0.1%) for Bcc isolated from environmental sources were significantly higher ($P < 0.05$) than MICs for Bcc from other sources. The mean MIC of sodium benzoate for Bcc from clinical sources (0.23% ± 0.15) was significantly lower ($P<0.05$) than MICs for Bcc from other sources.

Table 14. Bcc species and number of strains in clinical, environmental and environmental-industrial isolation source groups

	Number of strains from isolation source		
Species/group (number of strains tested)	Clinical	Environmental	Environmental industrial
B. ambifaria (4)	1	3	
B. anthina (2)		2	
B. arboris (4)	1		3
B. cenocepacia (15)	12	1	2
B. cepacia (4)	2	2	
B. contaminans (5)	2	1	2
B. diffusa (3)	2	1	
B. dolosa (3)	3		
B. lata (5)		1	4
B. metallica (2)	2		
B. multivorans (8)	6	1	1
B. pyrrocinia (3)		3	
B. stabilis (3)	2	1	
B. ubonensis (1)		1	
B. vietnamiensis (5)	2	1	2
BCC Kc (4)			4
BCC4 (3)	1	2	
BCC5 (3)	2	1	
BCC6 (3)	2	1	
BCC8 (1)	1		
unclassified BCC (2)		2	
Total number of strains	41	24	18

Table 15. The preservative susceptibility profile (MBC values) of 20 Bcc strains recommended for inclusion in future preservative efficacy tests

Species, Strain name	strain designation [2]	Isolation source code	MLST Sequence type	Preservative susceptibility profile by MBC (%)						
				MIT/CMIT	MIT	BIT	BC	DMH	PH	SB
B. arboris										
BCC1310	-	ENVI	327 [1]	0.0001124	0.00485	0.0015	0.05	0.324	0.5	2.5
B. cenocepacia III-A										
BCC0018 [ESP]	LMG 16659	CLIN	35	0.0001124	0.00194	0.0015	>1	0.081	0.5	1
BCC0560	-	CLIN	33	0.00075	0.00485	0.003	>1	0.081	0.5	2.5
B. cenocepacia III-B										
HI2424	-	ENV	122	0.00075	0.00485	0.006	>1	0.081	1	2.5
BCC1283 [PET*]	-	ENVI	250 [1]	0.0015	0.00485	0.003	>1	0.162	0.5	2.5
B. cepacia										
BCC0001 [ESP PET]	LMG 1222 [T]	ENV	10	0.00075	0.00194	0.006	0.05	0.081	0.5	2.5
BCC0002 [ESP]	LMG 2161	ENV	1	0.00075	0.00485	0.003	0.05	0.081	0.5	2.5
B. contaminans										
SAR-1	LMG 23255	CLIN	102	0.00075	0.00485	0.003	0.05	0.162	0.5	2.5
BCC1315	-	ENVI	341 [1]	0.00075	0.00485	0.003	0.05	0.162	1	2.5
B. diffusa										
BCC0169	(ATCC 29352)	ENV	108	0.00075	0.00194	>0.015	0.05	0.081	0.5	2.5
B. dolosa										
AU3556	-	CLIN	215	0.00075	>0.01	0.006	0.05	0.081	0.5	2.5
B. lata										
Strain 383	LMG 22485 [T]	ENV	101	0.0001124	0.00194	0.003	0.05	0.081	0.5	2.5
BCC1294	-	ENVI	98 [1]	0.00075	0.00194	0.003	0.1	0.081	0.5	1
BCC1296	-	ENVI	119 [1]	0.0001124	0.00485	0.015	0.05	0.081	0.5	1
B. multivorans										
ATCC17616 [ESP]	LMG 17588	ENV	21	0.00075	0.00485	0.003	0.05	0.081	1	2.5
BCC0390 [ESP]	LMG 18825	CLIN	15	0.00075	0.01	0.015	0.05	0.081	1	2.5
BCC1560	-	ENVI	439	0.0001124	0.00194	0.0015	0.05	0.324	0.5	2.5
B. vietnamiensis										
BCC0028 [ESP]	LMG 16232	CLIN	200	0.00075	0.00485	0.015	0.05	0.081	1	1
BCC1309	-	ENVI	326	0.00075	0.00194	0.003	0.05	0.081	1	2.5
BCC Kc										
BCC1282	-	ENVI	333 [1]	0.0001124	0.00485	0.003	0.2	0.162	0.5	1
BCC1313	-	ENVI	335 [1]	0.0001124	0.00194	0.003	>1	0.081	0.5	1

Footnotes: MBC, minimum bactericidal concentration. MIT, methylisothiazolinone; MIT/CMIT, methylisothiazolinone and chloromethylisothiazolinone; BIT, benzisothiazolinone; PH, phenoxyethanol; BC, benzethonium chloride; DMH, dimethylol dimethyl hydantoin; SB, sodium benzoate. BCCM/LMG, Belgian co-ordinated collections of micro-organisms, Ghent.; ATCC, American type culture collection ;BCC, Cardiff strain collection. CLIN, clinical; ENV, environmental; ENVI, environmental-industrial.; MLST, Multi locus sequence typing; [1] Strain included in MLST study of Bcc isolates from environmental-industrial sources. [T] Type strain; [ESP] *Burkholderia cepacia* complex experimental strain panel. [PET] Preservative efficacy testing challenge test strain; [PET*], isolate recovered during manufacturing process, an "in-house" challenge test organism.

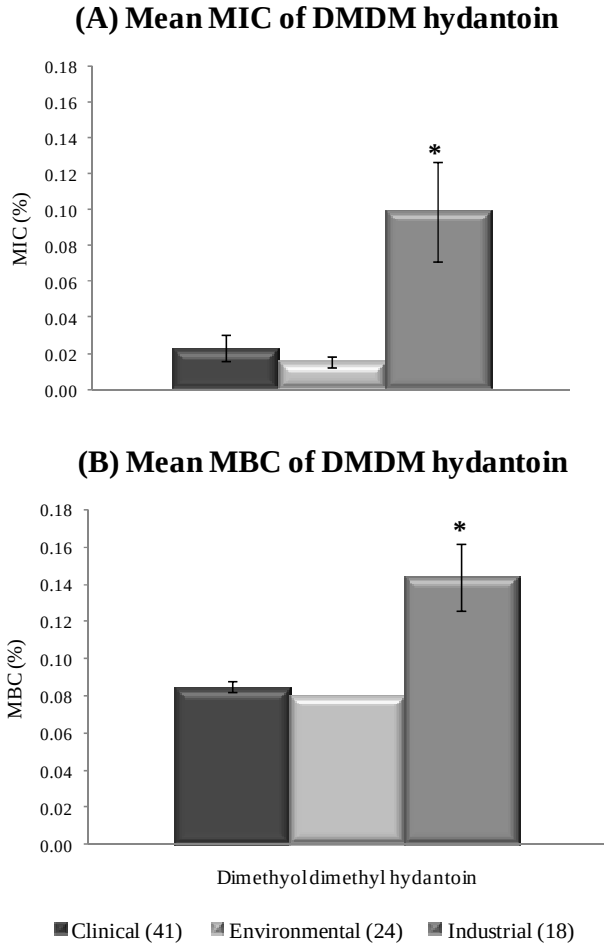


Figure 10. Increased tolerance to dimethylol dimethyol (DMDM) hydantoin in *Burkholderia cepacia* complex isolates from environmental-industrial sources.

The mean minimum inhibitory concentration (panel A) and mean minimum bactericidal concentration (panel B) of Bcc isolates from industrial sources were significantly higher than values of Bcc from clinical and environmental sources (* P = 0.000, Two-tailed Mann-Whitney test). In EU-regulated countries, a maximum level of 0.3% DMDM hydantoin is regulated for use in rinse-off personal care products (EU cosmetics directive 76/768/EEC Annex VI). The legend shows the isolation sources (number of strains). Error bars show standard error. Bcc species from clinical, environmental and environmental-industrial isolation sources are listed in Table 14.

3.2.4.4 Preservative susceptibility of non-Bcc bacteria reference strains

Preservative susceptibility of the 83 Bcc bacteria was also compared with a control group of ten non-Bcc species (Table 7). Bcc bacteria had significantly higher ($P < 0.05$) mean MIC (0.000133% ± 0.0001 v/v) and MBC (0.0004% ± 0.0004 v/v) values for the blend of isothiazolinone preservatives than the non-Bcc control group. The collection of Bcc strains also had significantly higher mean MICs for benzethonium chloride (0.148% ± 0.07 w/v) and sodium benzoate (0.27% ± 0.09 w/v) than the non-Bcc species group (mean MICs of 0.049% ± 0.07 w/v and 0.13% ± 0.09 w/v respectively). The non-Bcc species group had a significantly higher mean MBC for phenoxyethanol (1.3% ± 1.3 v/v) and MIC for methyl paraben (0.105% ± 0.114 w/v) than the Bcc species group (0.58% ± 0.19 v/v, and 0.093% ± 0.02 w/v respectively). There was no significant difference in the MIC or MBC of Bcc and non-Bcc bacterial species for methylisothiazolinone, benzisothiazolinone and DMDM hydantoin preservatives.

3.2.4.5 Activity of maximum permitted preservative concentrations

The collection of 83 Bcc strains and ten non-Bcc bacterial species were evaluated with preservatives at the maximum permitted concentrations for use in rinse-off personal care products in EU-regulated countries, as described in section 2.12.3. Additional concentrations above the maximum permitted levels of benzethonium chloride, DMDM hydantoin, sodium benzoate and phenoxyethanol were also evaluated. The highest concentration of benzisothiazolinone evaluated was based on the manufacturer's recommendation. MIC and MBC values were determined, using agar and broth dilution methods respectively. Six of the eight preservatives evaluated had strains with MIC and/or MBC values greater than the maximum permitted level of use in personal care products (Table 16). The maximum permitted levels of DMDM hydantoin (0.3%), phenoxyethanol (1%) and methyl paraben (0.4%) were most effective against the test organisms. All of the 83 Bcc strains were killed by maximum permitted levels of phenoxyethanol; all 93 strains (Bcc and non-Bcc strains) were killed by DMDM hydantoin.

Isothiazolinone preservatives efficiently inhibited and killed the majority of strains: maximum permitted levels were bactericidal to 91 (out of 93) strains. *B. dolosa strain* Bcc1356, BCC8 and *B. diffusa* strain Bcc169 had MBC values greater than the permitted levels of methylisothiazolinone (0.01%), the isothiazolinone blend (0.0015%) and

benzisothiazolinone (0.015%) respectively. The maximum permitted level of sodium benzoate (0.5%) effectively inhibited the growth of 80 Bcc strains, but was not high enough to kill the majority of Bcc strains in the collection; 76 had MBCs greater than 0.5%. Benzethonium chloride failed to inhibit growth of the majority (74 out of 83) of Bcc strains at maximum permitted levels of ≤ 0.1%. 13 Bcc strains, predominantly *B. cenocepacia* species, showed a high tolerance to benzethonium chloride, with MBCs up to ten times greater than the maximum permitted level.

The collection of ten non-Bcc strains had MBC values above the maximum permitted concentrations for six of the eight preservatives evaluated. The maximum permitted levels of phenoxyethanol (1%), bactericidal to Bcc strains, inhibited the growth of non-Bcc strains but was not bactericidal to *Enterococcus faecalis* and *P. fluorescens.* The former also had MBC values above the permitted levels for sodium benzoate and isothiazolinone preservatives.

3.2.5 Preservative combination testing

Pairwise comparisons of isothiazolinone preservatives in combination with other preservatives or preservative enhancing agents, were examined against *B. lata* strain 383 (as a model strain). The degree of interaction and interpretation of synergy testing (section 2.12.2.3), from the lowest FIC index of replicate plates, is shown in Table 17. All combinations have an additive (anti-Bcc) activity; none of the combinations evaluated had a synergistic outcome. The most efficacious combinations were methylisothiazolinone and EDTA, and the isothiazolinone blend with phenoxyethanol or EDTA; all had FIC indices of 0.8. EDTA (2% v/v), at 0.67 of the relative MIC, more than halved the concentration of isothiazolinone preservatives required to inhibit growth, to ≤ 0.14 of the relative MICs of individual agents (Table 17). A checkerboard approach in a Bioscreen growth analyser was used to examine growth dynamics of *B. lata* cultured in BSM (CYG) with combinations of methylisothiazolinone and methyl paraben, and the isothiazolinone blend with phenoxyethanol; lowest FIC indices for the respective combinations were 0.96 and 0.80. Growth curve analysis revealed that the duration of the lag phase increased with greater concentrations of the primary and/or secondary agent, resulting in a lower final optical density (450-580 nm) at 24 hours (data not shown). After an extended lag phase, the rate at which the optical density increased (i.e. the growth rate) was similar to that of the control

(without preservative); only combinations with higher concentrations of phenoxyethanol (0.15% v/v) had a slower rate of optical density increase than the control.

Table 16. Number of strains with MIC and MBC values greater than the maximum regulated concentration used in personal care products

Species/MLST group (number of strains evaluated)	Number of strains with MIC and MBC values above the regulated preservative concentration [1]															
	MIT		MIT/CMIT [2]		BIT [3]		BC		DDH		PH		MP		SB	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. ambifaria (4)	0	0	0	0	0	0	3	0	0	0	0	0	0	-	0	2
B. anthina (2)	0	0	0	0	0	0	2	0	0	0	0	0	0	-	0	2
B. arboris (4)	0	0	0	0	0	0	4	0	0	0	0	0	0	-	0	3
B. cenocepacia (15)	0	0	0	0	0	0	14	9	0	0	0 [a]	0	0	-	1	14
B. cepacia (4)	0	0	0	0	0	0	3	0	0	0	0	0	0	-	1	3
B. contaminans (5)	0	0	0	0	0	0	5	1	0	0	0	0	0	-	0	5
B. diffusa (3)	0	0	0	0	0	1	3	0	0	0	0	0	0	-	0	3
B. dolosa (3)	0	1	0	0	0	0	3	0	0	0	0	0	0	-	0	3
B. lata (5)	0	0	0	0	0	0	4	0	0	0	0	0	0	-	0	5
B. metallica (2)	0	0	0	0	0	0	2	0	0	0	0	0	0	-	0	2
B. multivorans (8)	0	0	0	0	0	0	8	0	0	0	0	0	0	-	0	8
B. pyrrocinia (3)	0	0	0	0	0	0	3	0	0	0	0	0	0	-	0	3
B. stabilis (3)	0	0	0	0	0	0	3	0	0	0	0	0	0	-	0	2
B. ubonensis (1)	0	0	0	0	0	0	1	0	0	0	0	0	0	-	0	1
B. vietnamiensis (5)	0	0	0	0	0	0	1	0	0	0	0	0	0	-	1	4
BCC Kc (4)	0	0	0	0	0	0	3	2	0	0	0	0	0	-	0	4
BCC4 (3)	0	0	0	0	0	0	3	0	0	0	0	0	0	-	0	3
BCC5 (3)	0	0	0	0	0	0	3	0	0	0	0	0	0	-	0	3
BCC6 (3)	0	0	0	0	0	0	3	1	0	0	0	0	0	-	0	3
BCC8 (1)	0	0	0	1	0	0	1	0	0	0	0	0	0	-	0	1
unclassified BCC (2)	0	0	0	0	0	0	2	0	0	0	0	0	0	-	0	2
non-Bcc (10)	0	1	0	1	0	1	3	1	0	0	0	2	0	-	0	3
Total number of strains (93)	0	2	0	2	0	2	77	14	0	0	0	2	0	-	3	79

Footnotes:

[1] The maximum level for use in rinse off personal care products according to EU cosmetics directive 76/768/EEC annex VI .

[2] A cosmetics grade commercial blend of methylisothiazolinone and chloromethylisothiazolinone preservatives

[3] Not permitted for use in personal care products in EU regulated countries, concentrations evaluated based on manufacturers recommended level .

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

Abbreviated preservative names and maximum in-use level : MIT, methylisothiazolinone 0.01%; MIT/CMIT ,methylisothiazolinone/ chloromethylisothiazolinone 0.0015% ; BIT, benzisothiazolinone 0.015%; BC, benzethonium chloride 0.1% ; DDH, dimethyl dimethol hydantoin 0.3%; MP, methyl paraben 0.4%; PH, phenoxyethanol 1%; SB, sodium benzoate. 0.5%

% refers to v/v for MIT, MIT/CMIT, PH and DMH; % refers to w/v for other agents.

[a] 14 *B. cenocepacia* strains evaluated .

MBC not determined for methyl paraben.

Table 17. Activity of preservative combinations against *B. lata* strain 383

Agents in combination	Concentration of agent in combination (%), Fraction of the relative MIC [1]					
	MIT	MIT/CMIT	DMH	PH	MP	EDTA
Primary (MIT)	8.63E-04	8.63E-04	3.78E-05	6.47E-04	6.47E-04	1.33E-04
	0.09	*1.00*	*0.04*	*0.67*	*0.30*	*0.14*
Secondary	7.57E-05	2.92248E-06	0.00405	0.44	0.022	2.0
	1.00	*0.09*	*1.00*	*0.30*	*0.67*	*0.67*
FIC index [2]	1.09	1.09	1.04	0.96	0.96	0.8
Relationship	Additive	Additive	Additive	Additive	Additive	Additive
Primary (CMIT)	2.92E-06	1.11E-05	1.1100E-05	2.2193E-05	2.2193E-05	4.9284E-06
	0.09	*0.30*	*0.30*	*0.67*	*0.67*	*0.13*
Secondary	8.63E-04	2.55E-05	0.00270	0.03	0.020	2.0
	1.00	*0.68*	*0.67*	*0.13*	*0.20*	*0.67*
FIC index [2]	1.09	0.98	0.96	0.80	0.86	0.80
Relationship	Additive	Additive	Additive	Additive	Additive	Additive

Footnotes:

MIT, methylisothiazolinone MIT/CMIT, methylisothiazolinone & chloromethylisothiazolinone blend; DMH, dimethyl dimethol hydantoin ; MP, methyl paraben ; PH, phenoxyethanol; EDTA, Ethylenediaminetetraacetic acid; MIC, minimum inhibitory concentration.[1] Fraction of the relative MIC of the individual preservative. [2] Fractional inhibitory concentration index or ∑FIC.

The relationship, or interpretation of synergy testing, was derived from the fractional inhibitory concentration index: a synergistic outcome defined as an $\sum FIC \leq 0.5$, additivity/indifference was defined as an $\sum FIC > 0.5$, antagonism was defined as $\sum FIC > 4$. The lowest FIC index of all non-turbid wells along the turbidity/non-turbidity interface of replicate plates was used.

3.2.6 Biofilm biomass production in the presence of sub-inhibitory preservative concentrations

A crystal violet assay was used to evaluate biofilm biomass formation of *B. lata* strain 383 and *B, multivorans* ATCC 17616 cultured in BSM (CYG) broth containing sub-inhibitory preservative concentrations as described in section2.14. Kruskal-Wallis and Mann-Whitney (one-tailed) statistical tests revealed that there was no significant increase ($P>0.05$) in biofilm biomass formation for strains cultured in BSM (CYG) broth containing preservative concentrations of ≤ 0.5 of the relative MIC (Figure 11). *B. multivorans* biofilm formation was significantly lower ($P <0.05$) when cultured in 0.5 of the relative MIC of all preservatives except sodium benzoate (Figure 11). In addition, *B. cenocepacia* C4455 and *B. multivorans* ATCC 17616 were cultured in BSM (CYG) with sub-inhibitory concentrations of the biocides chlorhexidine and CPC. There was no significant increase ($P >0.05$) in biofilm formation of either strain cultured in the presence of sub-inhibitory biocide concentrations (data not shown).

3.2.7 Swarming motility in the presence of sub-inhibitory preservative concentrations

Swarming motility is a type of social behaviour, characterised by the rapid migration of differentiated swarm cells on a semi-solid surface (Lai *et al.*, 2009). A modified swarming assay was used to investigate the effect of sub-inhibitory preservative concentrations on swarming motility in Bcc bacteria, as described in section 2.15. The swarming motility of *B. cenocepacia* J2315, *B. multivorans* ATCC 17616 and *B. lata* strain 383 on semi-solid swarm plates containing sub-inhibitory concentrations of preservatives (Figure 12) was not repressed or inhibited by concentrations lower than 0.5 of the relative MIC; swarming gradually decreased in correlation with increasing preservative concentration. Reduced swarming at 0.5 of the relative MIC was attributed to a slight inhibition of growth. In addition, sub-inhibitory concentrations of chlorhexidine and CPC biocides in semi-solid swarm agar failed to inhibit swarming motility of *B. multivorans.*

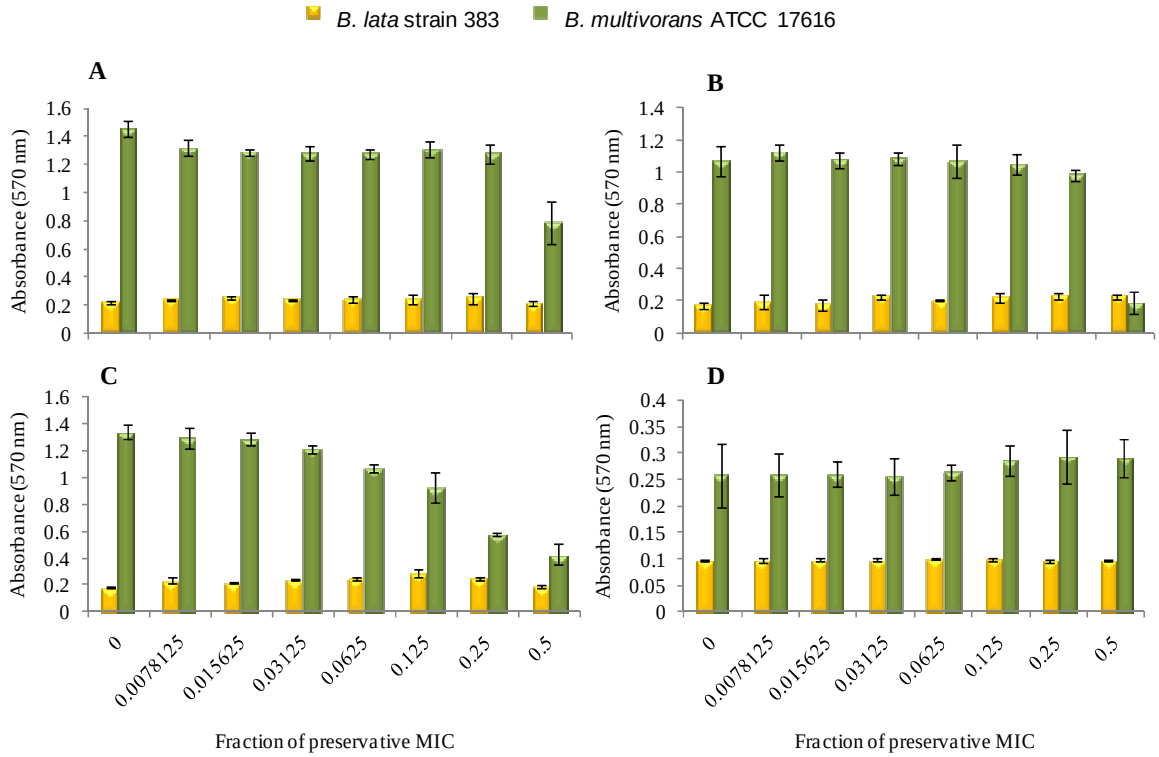


Figure 11. The effect of sub-inhibitory preservative concentrations on *B. lata* and *B. multivorans* biofilm production.

The average absorbance, obtained by crystal violet assay, of biofilms of *B. lata* strain 383 and *B. multivorans* ATCC 17616 cultured in BSM (CYG) broth containing sub-inhibitory concentrations of A) methylisothiazolinone B) benzisothiazolinone C) phenoxyethanol D) sodium benzoate. Preservative concentrations are presented as fractions of the relative MICs. Error bars indicate the standard deviation of the mean of triplicate stained wells of a test plate. Kruskal-Wallis and Mann-Whitney (one-tailed) statistical tests revealed no significant increase in biofilm production at sub-inhibitory concentrations of preservatives ($P > 0.05$).

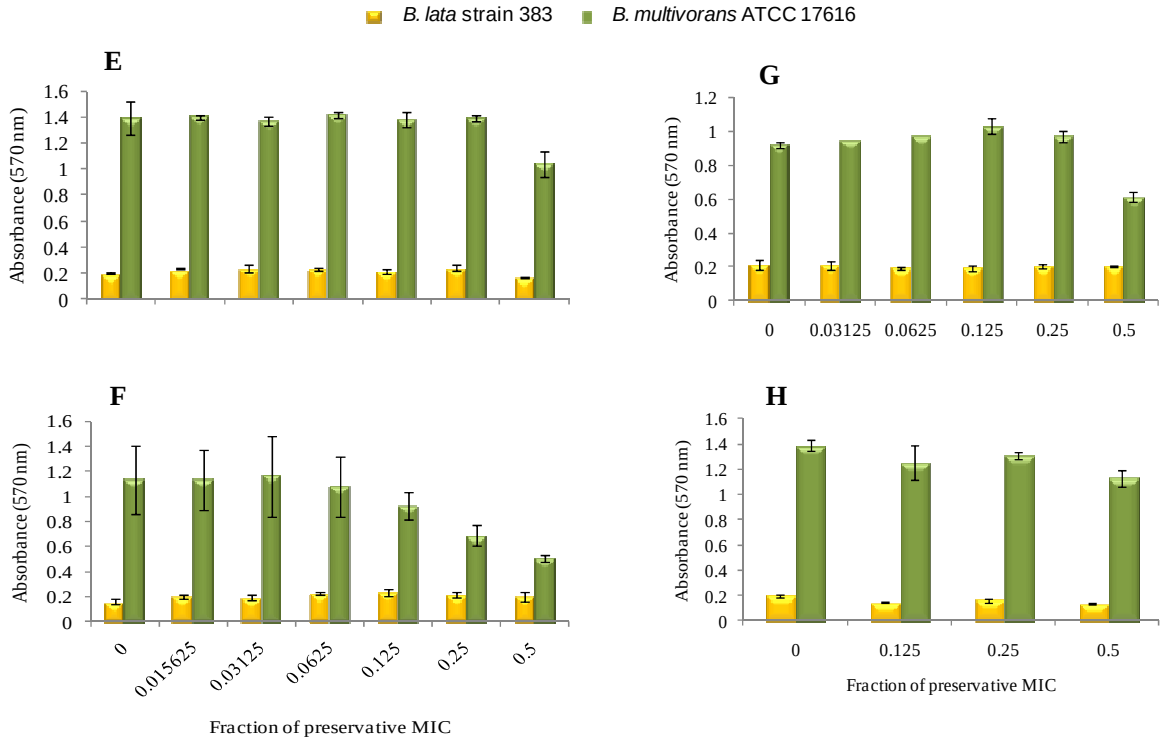


Figure 11. The effect of sub-inhibitory preservative concentrations on *B. lata* and *B. multivorans* biofilm production (continued).

The average absorbance, obtained by crystal violet assay, of biofilms of *B. lata* strain 383 and *B. multivorans* ATCC 17616 cultured in BSM (CYG) broth containing sub-inhibitory concentrations of E) DMDM hydantoin F) methyl paraben G) isothiazolinone blend H) benzethonium chloride. Preservative concentrations are presented as fractions of the relative MICs. Error bars indicate the standard deviation of the mean of triplicate stained wells of a test plate. Kruskal-Wallis and Mann-Whitney (one-tailed) statistical tests revealed no significant increase in biofilm production at sub-inhibitory concentrations of the preservatives evaluated ($P > 0.05$).

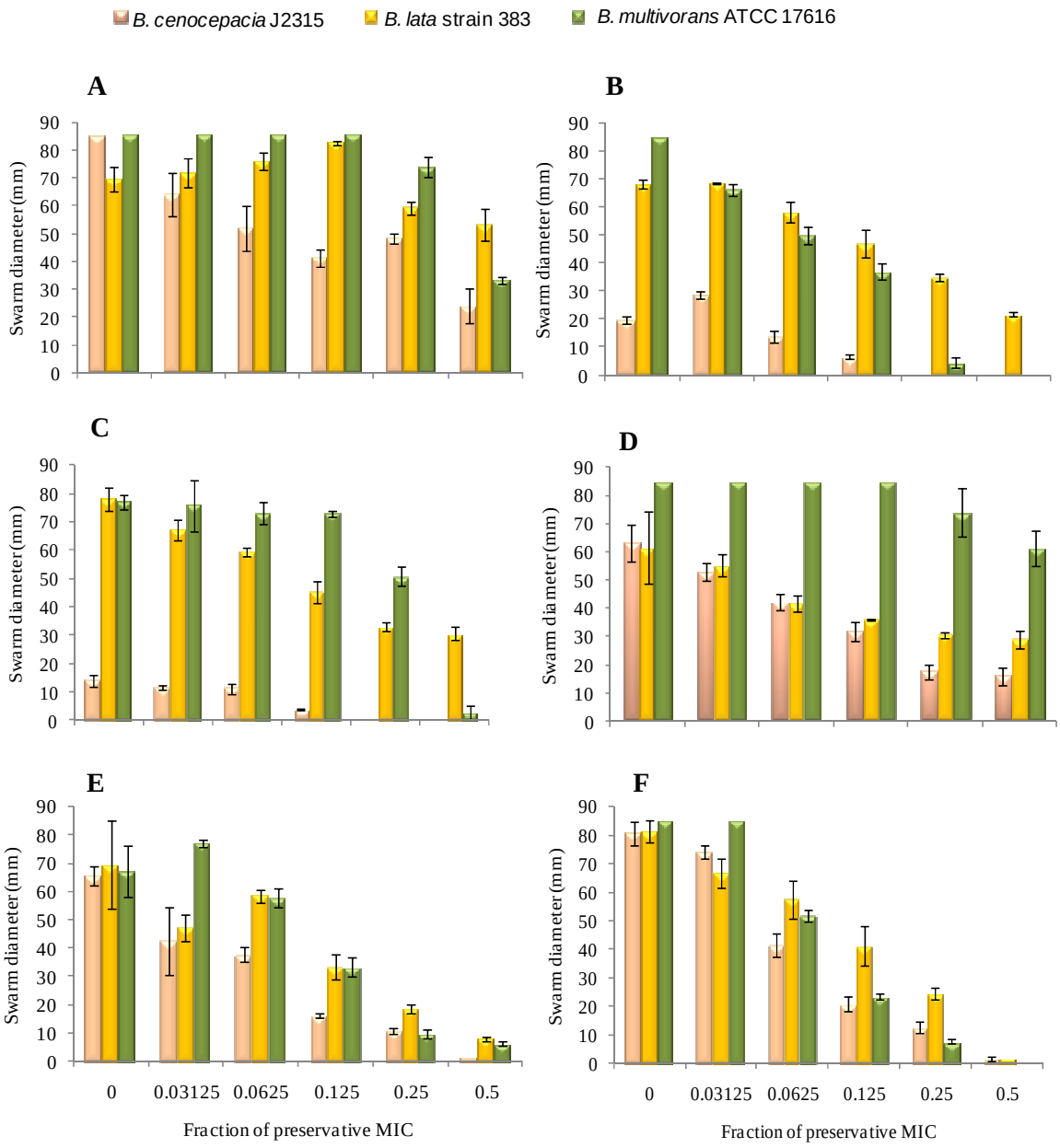


Figure 12. Swarming motility of *B. cenocepacia*, *B. lata* and *B. multivorans* in the presence of sub-inhibitory preservative concentrations.

The average swarm diameter (mm) of *B. cenocepacia* J2315, *B. lata strain* 383 and *B. multivorans* ATCC 17616 cultured in BSM (CYG) broth containing sub-inhibitory concentrations of A) methylisothiazolinone B) benzisothiazolinone C) DMDM hydantoin D) isothiazolinone blend E) methyl paraben F) phenoxyethanol. Preservative concentrations are presented as fractions of the relative MICs. Error bars indicate the standard error of triplicate experiments.

3.3 DISCUSSION

3.3.1 Bcc species diversity in the environmental-industrial niche

MLST analysis of a collection of 67 Bcc isolates from various environmental-industrial sources revealed that isolates belonged to nine formally named species or were novel sequence types. *B. lata* (25.4%) and *B. cenocepacia* (16.4%) were the most commonly encountered species groups in the collection. A low level of diversity was observed within the *B. lata* species group; sequence types were predominantly 98 or 119. Isolates with these sequence types generally shared a common geographical source of isolation (the exact source of isolation is confidential). Currently, there is little information on the prevalence of the recently formally named *B. lata* species in various ecological niches, although it is widely distributed, having been recovered from clinical, environmental and environmental-industrial sources. In contrast, *B. cenocepacia* is a well documented dominant CF pathogen, with *recA* lineages III-A, III-B and III-D accounting for the most transmissible and virulent strains (Speert *et al.*, 2002). Eight of the 67 environmental-industrial isolates (11.9%) were identified as *B. cenocepacia recA* lineage III-B. This subspecies is ubiquitous in the natural environment and is commonly associated with plant rhizospheres and aquatic environments (Mahenthiralingam *et al.*, 2008). Since water is the primary constituent of many industrial products (e.g. ≤80% of shampoos (Perry, 2001)), and is used in the washing of plant facilities, it is highly likely that industrial water supplies may be a potential source of *B. cenocepacia* III-B contaminants. Interestingly, three of the 67 environmental-industrial isolates (4.5%) were identified as belonging to *recA* lineage III-A. This subspecies is not commonly recovered from the natural environment; at present, its exact source and preferred habitat remain a mystery. Therefore, contaminated industrial product is one of the very few niches from which the subspecies has been recovered.

Other species groups encountered included *B. vietnamiensis, B. arboris, B. stabilis, B. cepacia, B. multivorans, B. contaminans* and *B. ambifaria.* Isolates from these species groups are widely distributed, having also been recovered from CF and non-CF infections and the natural environment (Mahenthiralingam *et al.*, 2008). Species distribution within the collection indicated potential differences in Bcc species distribution, and/or prevalence between industrial processes. Contaminants of industrial processes, such as petroleum and cosmetic products, may face very different environmental and metabolic challenges. It was unsurprising that *B. vietnamiensis* was the predominant Bcc species recovered from

petroleum products. The ability of *B. vietnamiensis* to degrade xenobiotics is well documented (O'Sullivan *et al.*, 2007); in 2010, White *et al.*, (2010) identified *Tom* pathway genes as being involved in the degradation of aviation kerosene in *B. vietnamiensis* strain JW13.1a. Genetic typing analysis of a larger collection of Bcc strains from various industrial processes would be required to identify significant differences in species distribution and prevalence between industrial processes, and may also identify species and sequence types widely distributed within the environmental-industrial niche.

Several studies have highlighted geographical differences in Bcc species distribution within environmental and clinical niches (Mahenthiralingam *et al.*, 2008; Vial *et al.*, 2011). For example, *B. ambifaria*, the most commonly encountered species group associated with maize cultivated in the USA and Italy (Dalmastri *et al.*, 2007; Ramette *et al.*, 2005), was not detected in the rhizosphere of maize cultivated in China, where *B. cenocepacia recA* lineage III-B was the dominant species recovered (Zhang & Xie, 2007). It is highly likely that similar geographical differences could occur within the environmental-industrial niche as the manufacture of industrial products occurs at various locations, often using locally sourced raw materials such as water. A recent study of 22 Bcc strains isolated from industrial products in Argentina, South America revealed *B. contaminans* (36%) and *B. lata* (36%) were the most commonly encountered species; *B. vietnamiensis* (18%), *B.cepacia* (5%) and *B. cenocepacia* (5%) were also identified (Degrossi, 2011). Geographical differences in Bcc species distribution and prevalence within the environmental-industrial niche could well have implications on the efficiency of current preservative efficacy and quality control tests. Preservative efficacy tests are conducted with recommended reference test organisms, such as *B. cepacia* (LMG 1222) and an “in-house” adapted organism isolated from raw materials and/or finished product (Orth *et al.*, 2006). It is not known whether manufacturers routinely tailor challenge test organisms to accommodate geographical or regional differences in species distribution. Preliminary reports of Bcc species distribution and prevalence in industrial and clinical sources in Argentina (Degrossi, 2011) suggest that future preservative efficacy test recommendations should include representative *B. contaminans* and/or *B. lata* challenge test organisms.

3.3.2 Preservative susceptibility testing

MIC and MBC values provide useful information about the concentration of preservative that may be required to protect industrial product from microbial insult. However, *in-vitro* susceptibility testing cannot predict the effect formulation constituents may have on preservative activity. In this study, preservative susceptibility testing was conducted *in-vitro* using a modified basal salts medium. The modified minimal medium better represented environmental-industrial conditions; was less detrimental to the recovery of stressed organisms; and was less likely to inactivate biocide activity than a complex medium (Chapman *et al.*, 1998). In order to evaluate accurately the anti-Bcc activity of individual preservatives, nitrilotriacetic acid was excluded from the medium, as chelating agents may enhance the activity of antimicrobial agents (Chopra, 1996). Modifications to the basal salts medium did not significantly alter the growth dynamics of Bcc bacteria, and did not influence turbidometric analysis. The lowest concentration of preservative resulting in an 80% reduction in optical density from the mean control (no preservative) was designated as the MIC when testing individual or combinations of preservatives, using a modified broth dilution method (Rose *et al.*, 2009). An 80% reduction was selected to allow for the increased opacity of some preservatives at higher concentrations that resulted in a "background noise" of turbidity that may be mistaken as bacterial growth. The opacity of preservative enhancing agents Ethylhexyl glycerin and caprylic acid in aqueous solution prevented evaluation by turbidometric analysis. Indicators, such as reasurin, could provide an alternative means of determining the end-point of MIC experiments for these compounds (Lambert & Pearson, 2000).

An efficient means of terminating or quenching the activity of preservatives that was non-toxic to the test organisms was required for MBC testing. This study adopted chemical neutralisation and dilution methods in an attempt to quench preservative activity. Dilution alone is not always sufficient at removing biocide bound strongly by chemical or electrostatic forces to viable cells (Johnston *et al.*, 2002). Higher test concentrations still inhibited growth after chemical neutralisation. An additional dilution step and an overnight culture of recovered cells from neutralised higher test concentrations successfully reduced active levels and/or removed residual active preservative, also facilitating the detection of viable cells. The efficacy of terminating activity of isothiazolinone preservatives could have been improved by

incorporating specific inactivators such as cysteine/thioglycollate into the neutralising solution (Denyer, 1995).

3.3.3 Preservative susceptibility varies between and within Bcc species

A recent survey of Bcc resistance to chlorhexidine, CPC, triclosan and povidine biocides demonstrated that levels varied widely across the complex; species-dependent differences for CPC were identified (Rose *et al.*, 2009). Interestingly, *B. cenocepacia*, the most prevalent species in clinical infection, generally demonstrated higher levels of tolerance to biocides and antibiotics (Rose *et al.*, 2009). The main aim of this study was to investigate the preservative susceptibility of a collection of genetically diverse Bcc bacteria isolated from clinical environmental and environmental-industrial sources; spanning the current 17 species groups and novel MLST Bcc groups. This study revealed that susceptibility to preservatives and preservative enhancers also varied considerably both between and within species and MLST groups. The prevalence of *B. lata* species in a collection of Bcc isolates from environmental-industrial sources (subjected to MLST analysis) could not be explained in terms of preservative susceptibility. None of the species or MLST groups demonstrated multi-preservative resistance (MPR). Statistical analysis of differences in susceptibility between groups was not examined, as over half of the groups had few (≤ 3) representative strains within the collection of 83 strains.

Preservative susceptibility of individual strains varied considerably within species and MLST groups of the collection. None of the Bcc strains had high broad-ranging tolerance to all preservatives evaluated. However, susceptibility profiling identified Bcc strains, such as *B.cenocepacia* strain Bcc1203, with high MIC and/or MBC values for unrelated preservatives with different cellular targets e.g. isothiazolinone and QAC. Cross-resistance to structurally unrelated compounds may indicate the involvement of generalised resistance mechanisms such as active efflux, alterations to the outer membrane and/or cellular permeability in preservative tolerance (Chapman, 1998; Chopra, 1996). Interestingly, differences in susceptibility to preservatives with a common mode of action were observed. Isothiazolinone preservatives predominantly target and oxidise thiol-containing cytoplasmic and membrane-bound enzymes; chloromethylisothiazolinone and benzisothiazolinone are thought to have a highly similar mode of action (Collier *et al.*, 1990a; Collier *et al.*, 1990b; Denyer, 1995).

Susceptibility profiles demonstrated individual Bcc strains with elevated tolerance to one or more isothiazolinone preservatives remained susceptible to preservative(s) of the same class. This may suggest that mechanistically similar agents may permeate the outer membrane via different routes.

Preservative susceptibility profiles of *B. dolosa* and *B. cenocepacia* strains with identical sequence types (by MLST analysis) varied by MIC and to a lesser extent MBC values. Both *B. dolosa* strains with ST-72 were clinical isolates from CF patients; *B. cenocepacia* strains AU1054 and H12424 (ST-122) were isolated from clinical (CF) and environmental settings respectively. Differences in susceptibility profiles are unsurprising as MLST analysis bases identity on sequence homology at seven loci, and as such cannot convey differences elsewhere in the genome or other adaptations that may alter the phenotype. Although indistinguishable by MLST, H12424 has a large genome of approximately 7.7 Mbp that includes a 164.9 Kbp plasmid; the clinical strain AU1054 has a smaller genome of ~7.2 Mbp and does not carry a plasmid (www.burkholderia.com). H12424 may possess additional resistance factors to AU1054.

3.3.4 DMDM hydantoin tolerance in Bcc from environmental-industrial sources

The collection of 83 Bcc strains used for susceptibility testing consisted of 41 clinical, 24 environmental and 18 environmental-industrial isolates. There were significant differences in preservative susceptibility in relation to the isolation source. Bcc bacteria isolated from environmental-industrial sources had significantly higher ($P < 0.0001$) mean MIC and MBC values for the formaldehyde releasing agent DMDM hydantoin. Previous exposure to formaldehyde releasing agents has been attributed to an increased tolerance in other bacterial species isolated from industrial sources. For example, industrial isolates of *P. aeruginosa* and *E. gervoviae* recovered from cosmetics or the floor of an industrial plant washing area were more tolerant of DMDM hydantoin than ATCC strains (Ferrarese *et al.*, 2003). Since the source of isolation is unknown, elevated tolerance to formaldehyde releasing agents in Bcc from environmental-industrial isolates cannot be easily explained.

Formaldehyde is a natural product of intermediate carbohydrate metabolism within cells; organisms may possess a means by which to detoxify such product. Resistance to

formaldehyde releasing agents, mediated by enzymatic systems, has been demonstrated in *P. aeruginosa, P. putida, Enterobacter spp.* and *B. cepacia* (Chapman, 2003a). Members of the Bcc potentially possess multiple pathways for formaldehyde detoxification (Marx *et al.*, 2004). Bcc tolerance to DMDM hydantoin is a common challenge to industrial manufacturers. Bcc challenge tests of DMDM hydantoin-preserved personal care products exhibit an initial decrease in viable cell count, often followed by a period where the organism is non-culturable, this is then followed by an increase in the number of viable cells recovered (Unilever, personal communication). The reason for this trend is currently unknown. It is possible that within the population a percentage of cells retain viability, entering a viable but non-culturable state (Oliver, 2005). It is unlikely that the emergent cells are from a sub-population of persister cells. These latter are a sub-population of cells that neither grow nor die in the presence of an antimicrobial (Keren *et al.*, 2004). However, upon removal of an antimicrobial agent, persister cells revert to a wild-type phenotype. Persisters exhibit tolerance but not an increased MIC, which normally results from the expression of specific resistance mechanisms (Keren *et al.*, 2004). It is unknown whether a reduction in activity levels of the formaldehyde releasing agent in the challenge test occurs prior to the re-emergence of viable cells. However, Bcc bacteria recovered from industrial sources had significantly higher tolerance to this agent than isolates from other sources. This strongly suggests that the selection of adapted tolerant organisms occurs in environmental-industrial settings. Borovian *et al.*(1983) reported a similar trend with formaldehyde preserved product challenged with Bcc bacteria (identified as *Pseudomonas cepacia*). Although lower levels of formaldehyde (200 ppm) added to products exhibited an initial activity, decreasing the CFU, the organisms grew to their original numbers within a short period of time. Adapted organisms recovered demonstrated a significant increased tolerance to killing, and cross-resistance to other antimicrobial agents (Borovian *et al.*, 1983).

The efficacy of formaldehyde-releasing preservative systems (against Bcc bacteria) in aqueous finished personal care products is significantly improved when combined with a chelating agent such as EDTA (personal communication, Unilever). Chelating agents such as EDTA disrupt the lipopolysaccharide structure in the outer membrane of Gram-negative bacteria, increasing cell permeability (Denyer, 1995). This suggests that the outer membrane may play an important role in Bcc tolerance to formaldehyde releasing agents. Perhaps a combination of Bcc resistance mechanisms results in a synergistic reduction in susceptibility (Chapman *et al.*, 1998). Low-level resistance to formaldehyde releasing agents, mediated by

a reduction in cellular permeability, can be increased when combined with the enzymatic inactivation of preservative agent that penetrates the permeability barrier. The mean elevated MIC and MBC values of DMDM hydantoin for environmental-industrial Bcc isolates were still below the maximum concentration permitted for use in personal care products. However, any reduction in susceptibility is of major concern and may have commercial consequences, as the susceptibility of Bcc bacteria in the natural environment would be further reduced by their presence within biofilms (Chapman, 1998).

3.3.5 Are Bcc bacteria more preservative-tolerant than non-Bcc bacterial species?

Preservative susceptibility of Bcc bacteria was compared to a collection of ten non-Bcc bacterial species. The collection of non-Bcc bacteria included multi-drug resistant reference strains; and clinical, environmental and environmental-industrial isolates. *Acinetobacter* spp., *P. fluorescens* and *P. putida* are challenge test organisms recommended by the Cosmetics, Toiletry and Fragrance Association (CFTA) (Orth *et al.*, 2006). Bcc had a higher tolerance to isothiazolinone preservatives, benzethonium chloride and sodium benzoate, but were more susceptible to phenoxyethanol and methyl paraben. Significant differences in susceptibility were not due to the preservative spectrum of activity, as the non-Bcc bacterial strains like Bcc bacteria were predominantly Gram-negative; *E. faecalis* was the only Gram-positive organism evaluated. Paraben and benzoic acid preservatives are thought to be mechanistically similar, disrupting the proton motive force at the cell membrane, and causing the acidification of the cell cytoplasm (Maillard, 2002). Phenoxyethanol also targets the cell membrane by dissipating the proton motive force, but also inhibits membrane bound enzymes. Higher tolerance to sodium benzoate, but susceptibility to mechanistically similar agents, may be as a result of experimental conditions and/or an innate tolerance to aromatic compounds. Susceptibility testing of sodium benzoate activity was conducted at pH 5, as the agent has lower activity in neutral to alkaline conditions. Bcc bacteria have a high tolerance to acidic conditions and have reportedly been recovered from formulation with a pH of $\leq$3 (Borovian *et al.*, 1983). In addition, members of the Bcc are well known for the capacity to degrade aromatic xenobiotic compounds (Denef, 2007). Predicted metabolic pathways for the degradation of benzoate can be found in numerous Bcc genomes, including *B. lata* strain 383, *B. cenocepacia* AU1054, and *B. multivorans* ATCC17616 (http://genome.ornl.gov/microbial/).

3.3.6 Anti-Bcc activity of individual preservatives at maximum permitted concentrations

The use of preservative agents in personal care products is strictly regulated, especially in countries of the European Union and Japan. Attached to the European Directive 76/768/EEC are annexes which contain lists of preservatives which cosmetic products may or may not contain, as well as the permitted active levels of preservatives in the finished product. In this study, a collection of 83 Bcc bacteria were evaluated with preservatives at the maximum permitted concentrations for use in rinse-off personal care products, as outlined in the EU directive 76/768/EEC. The maximum permitted levels of DMDM hydantoin, phenoxyethanol and methyl paraben were most effective against Bcc bacteria. Although Bcc isolates from environmental-industrial sources were significantly more tolerant to DMDM hydantoin than Bcc from other sources, all Bcc strains were killed by the maximum permitted level of 0.3%.

Due to the allergenic properties of chloromethylisothiazolinone, it is only regulated for use at low-levels when blended with methylisothiazolinone. Even at low levels, the blend of isothiazolinone preservatives was effective against Bcc bacteria. Only one clinical isolate, from the novel MLST group BCC8, had an MBC greater than the maximum permitted level. Methylisothiazolinone and benzisothiazolinone also had excellent anti-Bcc activity, killing all Bcc except clinical isolates of *B. diffusa* and *B. dolosa* respectively. Maximum permitted levels of benzethonium chloride failed to inhibit the majority of Bcc bacteria when evaluated using agar dilution methods. However, using a broth dilution method the maximum levels of benzethonium chloride were lethal to the majority of Bcc strains.

Agar and broth dilution methods evaluate the susceptibility of sessile and planktonic organisms respectively. Susceptibility profiling is influenced by many factors (Lambert & Pearson, 2000). Differences may be attributed to interaction with a preservative agent in a solid and liquid medium. The few (n =11) Bcc strains with MBCs up to ten times greater than the permitted level were predominantly *B. cenocepacia*, a species known to possess high levels of resistance to both biocides and antibiotics (Mahenthiralingam *et al.*, 2005). This species group also had the largest number (15) of representative strains within the collection, which may have biased the findings. However, other species groups with ≥ five representative strains (*B. lata, B. multivorans and B. vietnamiensis)* were susceptible to maximum permitted levels; only one *B. contaminans* strain had an elevated MBC value. The

least effective preservative at permitted levels was sodium benzoate, failing to kill the majority of Bcc (as discussed in section 3.3.5).

3.3.7 Anti-Bcc activity of preservative combinations

A single preservative agent is often inadequate protection for an industrial process that may face multiple challenges of microbial contaminants during manufacture and consumer use. Preservative systems are carefully designed to complement, and function within, complex product formulation, providing a spectrum of antimicrobial activity. Synergistic or super-additive combinations of preservatives have several additional advantages. A reduction in concentration of individual agents may reduce the cost of manufacturing and prevent the withdrawal of effective preservatives from the market due to allergenic reactions (Lundov *et al.*, 2011). This study evaluated the interaction of preservatives and enhancers, frequently used in personal care products, with isothiazolinone preservatives; identifying combinations with potential synergistic or super-additive anti-Bcc activity. The lowest FIC index of all the non-turbid wells along the turbid/non-turbid interface was used to define the outcome of an interaction. As this method of interpretation is reported to have the highest likelihood of detecting synergy (Bonapace *et al.*, 2002), synergy was strictly defined as an $\sum FIC \leq 0.5$ (Eliopoulos, 1996).

Combinations of isothiazolinone preservatives with EDTA, phenoxyethanol, methyl paraben and DMDM hydantoin all had FIC indices just below 1, indicating an additive effect. Combinations of two preservatives at low concentrations were just as effective as a high concentration of a preservative alone. The best combinations, resulting in the lowest reproducible FIC index (~0.8), were observed when isothiazolinone preservatives were combined with phenoxyethanol or EDTA. Both agents disrupt the barrier properties of the cytoplasmic membrane. In both cases, this promotes permeation of the isothiazolinone preservative. None of the combinations evaluated resulted in a synergistic outcome. EDTA, a chelating agent, has been shown to enhance or potentiate the activity of numerous antimicrobials (Denyer *et al.*, 1985; Lambert *et al.*, 2004). The interaction of other reported enhancing agents, such as caprylyl glycol and ethylhexyl glycerine, with isothiazolinone agents, could not be evaluated by turbidometric analysis, due to the opacity of agents in aqueous solution.

The dose response of many antimicrobials is non-linear; yet the analysis of combinations for synergy is based on the linear addition of individual effects of agents(Lambert *et al.*, 2003); the ∑FIC linear model is only applicable to mixtures of antimicrobials which individually have similar dose responses (dilution coefficient or concentration exponent) (Lambert *et al.*, 2003). The simple linear additive model ignores the concept of the dilution coefficient and describes the phenomenon of synergy for combinations where one or more of the components have a dilution coefficient greater than 1 (Johnston *et al.*, 2003). There are currently no published lists that define the dilution coefficient for all of the preservatives evaluated in this study. However, published coefficients for alcohols (ŋ = 10) parabens (ŋ = 2.5), quaternary ammonium compounds (ŋ = 1) and formaldehyde (ŋ = 1) (Russell, 2004b) agents would suggest the linear model used in this study may produce an erroneous interpretation of the interactions between compounds. Ultimately, the activity of favourable preservative combinations should be evaluated in the product to be preserved, as in vitro analysis of interactions may not truly reflect potential results in vivo; ingredients and formulation design have been shown to influence the potential for synergistic activity (Denyer *et al.*, 1985).

3.3.7.1 Sub-inhibitory preservative concentrations did not induce biofilm formation or inhibit swarming motility in *B. lata* strain 383 or *B. multivorans* ATCC 17616.

Biofilm and swarming motility assays, which revealed sub-inhibitory concentrations of seven preservative agents, did not induce biofilm formation or inhibit swarming motility in *B. lata* strain 383 or *B. multivorans* ATCC 17616. The evaluation of a collection of genetically diverse Bcc is required to characterise fully phenotypic adaptation in response to preservative agents, identifying species or strain specific differences. Sub-inhibitory concentrations of biocides chlorhexidine and CPC, shown to induce biofilm formation and inhibit swarming motility in a previous study (Rose, 2009), failed to elicit the same response in *B. cenocepacia* C4455 and *B. multivorans* when assayed in model systems used in this study. This may be attributed to differences in the protocol for determining biofilm biomass production and swarming motility. In this study, microtitre plates used to evaluate biomass production were not coated in porcine mucin; a technique used to aid adherence of bacterial cells. Staining of the biofilm biomass was performed using a ten-fold lower concentration of crystal violet (0.1% v/v), as described by Peeters *et al* (2008a), to improve resolution and reproducibility

between replicates. Swarming assays were performed using a semi-defined medium (ISO), not a general purpose medium (NB) supplemented with additional glucose (0.5%). Differences in the phenotypic response of test organisms may have been a result of using different stock cultures of *B. multivorans* ATCC 17616 and *B. cenocepacia* C4455. Identical isolates of *B. multivorans* ATCC 17616 have been shown to suffer genomic deletions and subsequent phenotypic alterations (Mahenthiralingam *et al.*, 2000b). Agnoli *et al.* (2011), reported the loss of chromosome three in *B. cenocepacia* strains and *B. lata* strain 383 occurred at a remarkably high frequency in culture without selective pressure (~1 in 1000 on LB medium), the resultant strains demonstrating an altered phenotype. Bcc strains used in this study may have suffered a genomic deletion or alteration that affected the regulatory response to the presence of chlorhexidine and CPC biocides. However, bacterial stocks were stored frozen, and not passaged, suggesting such major changes were unlikely.

Over the past decade, many studies have been conducted to elucidate the regulatory mechanisms involved in the transition from motility to sessility and biofilm formation (Cotter & Stibitz, 2007; Karatan & Watnick, 2009). Quorum sensing (QS), two component regulatory systems and secondary signalling molecules such as cyclic diguanosine monophosphate (c-di-GMP), have been implicated in the regulation of sessility in Gram negative bacteria (Daniels *et al.*, 2004; Karatan & Watnick, 2009; Simm *et al.*, 2004). Inhibiting the transition from motile to non-motile, and the associated increase in resistance to antimicrobials, make such regulatory systems an attractive target. The CepI/CepR system is a well characterised QS system in Bcc bacteria which is shown to regulate expression of target genes involved in diverse functions including swarming motility, and the maturation of the biofilm (Eberl, 2006; Huber *et al.*, 2001). Studies indicate that all Bcc species contain the CepI/CepR system and utilise N-acyl-homoserine lactone (AHL) signal molecules to express certain phenotypic traits in a population density dependent manner (Eberl, 2006). Additional QS systems, which potentially interact with the CepI/CepR system, have also been identified in Bcc species (Eberl, 2006). A study of the anti-biofilm effect of several well-known QS inhibitors in Bcc bacteria suggests that many compounds (at sub-inhibitory concentrations) do not affect the initial stages of attachment during biofilm formation, but rather promote the detachment of later stages (Brackman *et al.*, 2009). Whether quorum-sensing inhibitors, used alone or in combination with conventional antimicrobials, will ever be useful anti-biofilm agent remains to be determined (Brackman *et al.*, 2009).

3.3.8 Preservative susceptibility testing strain recommendation

In order to determine whether raw materials and finished industrial products are adequately preserved they are challenged by exposure to specific types of bacteria and fungi representative of organisms likely to occur as contaminants during manufacture and consumer use (Russell, 2003a). Challenge test organisms are inoculated, as a single or mixed inoculum, into samples of the test material and are periodically sampled at appropriate intervals to determine survivors (Orth *et al.*, 2006; Russell, 2003a). This study revealed four important findings that may impact on preservative efficacy testing, *viz.*

(1) preservative susceptibility varies considerably both between and within Bcc;

(2) environmental-industrial isolates are significantly more tolerant to certain preservatives than Bcc deposited in national culture collections (from environmental or clinical sources);

(3) *B. lata* and *B. cenocepacia* are commonly encountered species in the environmental-industrial niche;

(4) there may be geographical differences in the distribution and prevalence of Bcc species encountered in industry.

Microorganisms generally recommended for use in various challenge test methods are those deposited in national culture collections; the actual identity of strains recommended for use in methods such as CTFA are not identified. In addition, a few preservative efficacy testing guidelines recommend the use of in-house microbial strains obtained from either environmental sampling or contaminated product (Orth *et al.*, 2006). This study has demonstrated the importance of using in-house test strains, as environmental-industrial isolates are significantly more tolerant, to preservatives such as DMDM hydantoin, than Bcc deposited in national culture collections (from environmental or clinical sources). *Burkholderia cepacia* (strain not defined) and *B. cepacia* ATCC 25416 (LMG 1222) are typically recommended for use in the United States Pharmacopeia (USP) test method USP-29 and CTFA methods M-3/M-4 respectively (Orth *et al.*, 2006). MLST analysis of a collection of Bcc isolates from environmental-industrial sources demonstrated that *B. cepacia* were perhaps not a predominating species; *B. lata* and *B. cenocepacia* were the most commonly encountered species groups. Are recommended *B. cepacia* strains a good representation of Bcc bacteria encountered in industry? Firstly, current recommended Bcc challenge test organisms fail to represent the diversity of Bcc species encountered in industry, and any geographical differences in the prevalence of Bcc species. In addition, preservative susceptibility varies between and within Bcc species. The varied preservative susceptibility of

the recommended *B. cepacia* species group itself has important implications for methods that do not specify a specific test strain.

Based on the findings of this study, Bcc strains recommended for inclusion in future challenge tests are shown in Table 15. This panel offers genetically diverse representative strains from species commonly encountered in environmental-industrial sources. Selection and inclusion of strains in challenge tests could be tailored to represent prevalent species and/or sequence-type of a region. All strains selected have an elevated preservative tolerance (high MBC value); *B. arboris* (BCC 1310) and *B. multivorans* (BCC 1560) were the only strains selected for elevated tolerance to DMDM hydantoin. Where appropriate, a national culture collection strain and an environmental-industrial isolate were selected to represent the species group. Common sequence types encountered in a collection of environmental-industrial isolates were included. *B. dolosa* and *B. diffusa* strains with abnormally high tolerance to isothiazolinone preservatives were also included; these species have not been isolated from environmental-industrial sources to date.

3.4 CONCLUSIONS

The main conclusions from this chapter are as follows:

1) *B. lata* and *B. cenocepacia* were the most commonly encountered Bcc species in a collection of Bcc isolates from environmental-industrial sources. Further genetic typing studies are required to explore differences in the distribution and prevalence of Bcc species between industrial processes, geographical locations, and to identify widely distributed prevalent sequence types in industry. Industrial epidemiology would ultimately enable manufacturers to better target these organisms and would greatly enhance our understanding of Bcc species diversity in relatively unexplored ecological niches.

2) Preservative susceptibility varied considerably both between and within Bcc species groups. Species groups commonly encountered in a collection of Bcc isolates from environmental-industrial isolates were not highly tolerant to multiple preservative agents. Bcc from environmental-industrial sources were significantly more tolerant to DMDM hydantoin, suggesting extensive use of the agent in industrial processes selects for highly tolerant Bcc bacteria.

3) Isothiazolinone, DMDM hydantoin, phenoxyethanol and methyl paraben preservatives were highly effective against Bcc bacteria when evaluated at the maximum concentration for use in rinse-off personal care products in EU-regulated countries.

4) Benzethonium chloride and sodium benzoate had the weakest anti-Bcc activity at maximum regulated levels.

5) Combinations of isothiazolinone preservatives with other preservative agents and EDTA resulted in an additive anti-Bcc activity. The greatest anti-Bcc activity was observed when methylisothiazolinone was combined with EDTA, and when the blend of methylisothiazolinone and chloromethylisothiazolinone preservatives was combined with phenoxyethanol or EDTA. None of the combinations were interpreted as having synergistic or antagonistic activity against Bcc bacteria.

6) Sub-inhibitory concentrations of preservatives agents did not induce the transition from motility to sessility in *B. lata* and *B. multivorans* strains. Further screening of a genetically diverse collection of Bcc strains is required to determine whether the response and phenotypic adaptation to preservatives is agent-and/or species-specific.

4 ADAPTIVE RESISTANCE TO PRESERVATIVES

4.1 INTRODUCTION

To date the most extensively studied mechanisms relate to intrinsic and acquired resistance. In contrast, the phenomenon of adaptive resistance has been relatively poorly studied. Adaptive resistance can be defined as the induction of resistance to one or more antimicrobial agents in response to the presence of a specific signal, therefore, resistance increases due to the conditions under which the bacterium is growing (Fernández *et al.*, 2011; Russell, 2004a). The stability of adaptive resistance to antimicrobials is somewhat controversial and until recent years was not considered to play a role in the development of resistance. Historically, adaptive resistance was often characterised as transient; however, several studies now suggest the level of increased resistance and the duration it persists, may depend on the antimicrobial agent, dose, time of exposure and bacterial species (Fernández *et al.*, 2011; Russell, 2004a). Therefore, resistance may not completely revert to wild-type levels upon removal of a stimulus.

The development of biocide-induced adaptive resistance is not a new phenomenon, and has been documented in several bacterial species for a number of chemical agents including several preservatives (Orth & Lutes, 1985). For example, adaptive resistance to benzalkonium chloride, phenoxyethanol and isothiazolinone biocides has been promoted in *Pseudomonas spp.* via progressive sub-culture in media containing sub-inhibitory concentrations of an agent (Abdel Malek & Badran, 2010; Brozel & Cloete, 1994; Joynson *et al.*, 2002; Sondossi *et al.*, 1999). Although not yet fully understood, the mechanisms leading to biocide-induced adaptive resistance are considered diverse. Non-specific resistance mechanisms (e.g. outer membrane protein alterations and efflux) are considered important in biocide-induced adaptive resistance, as generally biocides have multiple target sites within a cell (Denyer, 1995). As a result, cross-resistance to other biocides and antibiotics are phenomena commonly encountered in experiments of adaptation (Fernández *et al.*, 2011). Cross-resistance profiles of biocide-adapted strains have been shown to vary, suggesting the mechanisms leading to biocide-induced adaptive resistance can be agent and/or strain specific (Braoudaki & Hilton, 2004; Loughlin *et al.*, 2002).

Biocides are used extensively in industry for plant sanitation and as preservative agents of raw materials and finished products. Although these agents are normally used at lethal or inhibitory concentrations, within the environment there is likely to be a gradient of

concentrations ranging from bactericidal to sub-inhibitory (Gilbert & McBain, 2003). Preservatives of personal care products are used at concentrations considerably lower than that of disinfectants; as a result, the risk of exposure to sub-inhibitory concentrations of preservatives can be considered greater than that of disinfectants. Exposure to sub-inhibitory preservative concentrations may occur as a result of the inadequate sanitation of production lines i.e. a dilute product residue retained in the process stream; the degradation of a preservative system by contaminating microorganisms; the leaching or binding of preservative agents; or the detrimental activity of formulation excipients on the activity of a preservative system (Orth *et al.*, 2006; Orth & Lutes, 1985). Contaminating bacteria, exposed to sub-inhibitory concentrations, may have the potential to develop biocide-induced adaptive resistance that may contribute to the acquisition of additional resistance traits, and the development of high-level resistance. Borovian *et al.* (1983) detailed the adaptation of *B. cepacia* (*P. cepacia)* to preservatives in dilute product formulation. Following exposure of the *B. cepacia* isolate (recovered from product formulation) to sub-inhibitory concentrations of formaldehyde in dilute product, the adapted-isolate displayed high-levels of resistance to formaldehyde and cross-resistance to benzoic acid.

Although members of the Bcc are frequently encountered as contaminants of preserved industrial processes, their adaptation to preservatives has not been extensively studied. The identification of any preservatives that are recalcitrant to Bcc adaptation, and those that may lead to the development of MPR or cross-resistance to other antimicrobials, would be of considerable benefit to manufacturers.

4.1.1 Aims

The aims of this chapter were as follows:

1) to investigate the potential for Bcc adaptive resistance to individual preservative agents used in industrial processes, via the progressive subculture of *B. lata* strain 383 on media containing increasing sub-inhibitory concentrations;

2) to determine the susceptibility of preservative-adapted derivatives of *B. lata* strain 383 to individual preservative agents, using a broth microdilution assay; thereby, identifying which agents promote the induction of resistance mechanisms that may lead to preservative cross-resistance;

3) to examine the susceptibility of adapted derivatives of *B. lata* strain 383 to antibiotics, using Etest® strips and a broth microdilution assay; thereby, identifying which agents promote the induction of resistance mechanisms that may lead to antibiotic cross-resistance;

4) to examine the stability of preservative-induced resistance of *B. lata* strain 383 (i.e. whether levels of resistance revert to wild-type levels upon removal of the priming preservative), via the sub-culture of adapted derivatives in the absence of preservative, with a subsequent re-evaluation of susceptibility (as previously described in aims 2&3);

5) to characterise the growth dynamics of the adapted derivatives of *B. lata* strain 383 in the absence and presence of individual preservative agents, using a Bioscreen C automated plate reader;

6) to examine whether preservative-induced adaptive resistance of *B. lata* strain 383 alters swarming motility;

7) to establish whether preservative-induced adaptive resistance of *B. lata* strain 383 alters biofilm formation, by quantifying the biofilm biomass production of adapted derivatives, in the absence, and presence of preservatives, using a crystal-violet staining assay.

4.2 RESULTS

4.2.1 Adaptive resistance of *B. lata* strain 383 to preservatives used in industry

Four derivatives of *B. lata* strain 383 were isolated as a result of its progressive subculture on agar containing increasing sub-inhibitory concentrations of preservatives; the isolated derivatives consistently grew at preservative concentrations higher than that of the parental strain. Adaptive resistance had been developed to methylisothiazolinone, the blend of methylisothiazolinone and chloromethylisothiazolinone (MIT/CMIT), benzisothiazolinone (BIT) and benzethonium chloride (BC) preservatives. The preservative-adapted derivatives were each assigned a suffix denoting the priming preservative that had induced adaptation (i.e. *B. lata* 383-MIT, 383-CMIT, 383-BIT and 383-BC). An increased tolerance to methyl paraben, DMDM hydantoin and phenoxyethanol, was not developed by stepwise training using an agar dilution method.

Following isolation, the identity of preservative-adapted *B. lata* strain 383 derivatives was confirmed by RAPD analysis. RAPD profiles of the preservative-adapted derivatives and the parental strain are shown in Figure 13. The digitalised bioanalyser fingerprint and clustering analysis demonstrated the preservative-adapted derivatives were *B. lata* strain 383: RAPD profiles were > 94.8% similar to the parental strain.

Phenotypic analysis demonstrated that the preservative-adapted derivatives did not have an altered cellular or colony morphology (data not shown); however, the BC-adapted derivative produced a higher intensity of blue-green pigment in confluent growth areas on BSM (CYG) agar, and in BSM (CYG) broth cultures after 18 hours of incubation.

4.2.2 Preservative susceptibility of adapted *B. lata* derivatives

4.2.2.1 MICs of preservatives determined by broth microdilution assay

A modified microdilution broth assay was used to determine the susceptibility of *B. lata* strain 383 (wild-type) and preservative-adapted derivatives to seven preservatives used in industry: the minimum inhibitory concentrations and fold-change from wild-type levels are shown in Table 18. The MIC values for adapted derivatives did not exceed the maximum regulated levels for use in personal care products in EU-regulated countries (see Table 2 for levels).

Preservative susceptibility profiles varied between adapted derivatives. *B. lata* 383-CMIT and 383-BIT both demonstrated a four-fold increase in MIC values for the MIT/CMIT blend and benzisothiazolinone; however, *B. lata* 383-BIT displayed a two-fold increased susceptibility to benzethonium chloride. The adapted derivative *B. lata* 383-BC demonstrated a two-fold increase in tolerance to benzethonium chloride and benzisothiazolinone agents, but a decreased tolerance to methylisothiazolinone, methyl paraben and phenoxyethanol (Table 18). The preservative susceptibility profile of the isothiazolone-adapted derivative 383-MIT was similar to that of the wild-type, the only difference being an increased susceptibility (two-fold) to benzethonium chloride (Table 18). Interestingly, both isothiazolone and benzethonium chloride adapted derivatives did not demonstrate an altered susceptibility to DMDM hydantoin, or an increased tolerance to phenoxyethanol and methyl paraben preservatives (Table 18).

Susceptibility profiles suggested that the mechanisms leading to MIT/CMIT and BIT-induced adaptive resistance conferred cross-resistance between the related compounds: isothiazolone-adapted derivatives 383-CMIT and 383-BIT each demonstrated a four-fold increase in MIC values for MIT/CMIT and benzisothiazolinone. In addition, the susceptibility profile of the benzethonium chloride-adaptive derivative 383-BC suggested cross-resistance to the unrelated compound benzisothiazolinone, as the MIC for both agents had increased two-fold (Table 18).

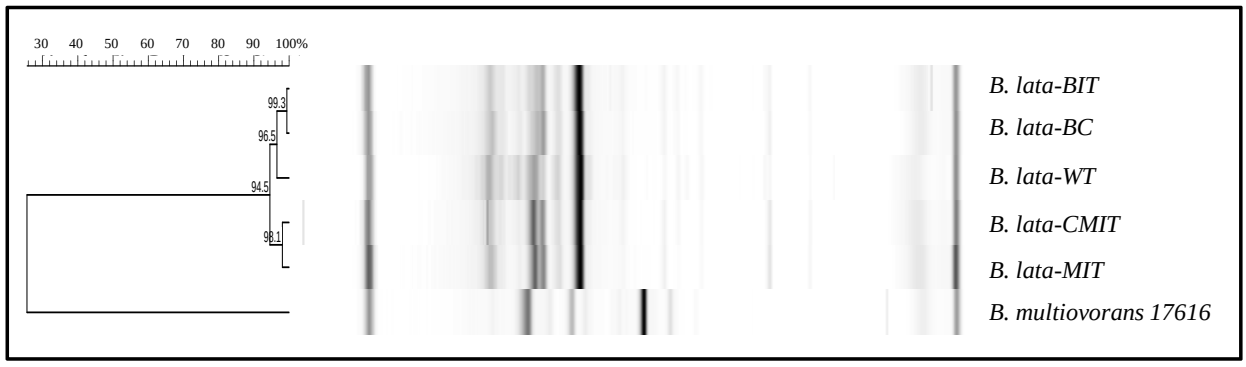


Figure 13. RAPD profile analysis of *B. lata* strain 383 wild-type and preservative-adapted derivatives.

Digitalised Bioanalyser fingerprinting profiles and clustering analysis were performed in GelCompar (Applied Maths). Pearson correlation similarity coefficient with UPGMA dendrogram type was used and position tolerance optimisation was set at 0.5%. Preservative adapted derivatives 383-CMIT, 383-MIT, 383-BIT and 383-BC were >94.5% similar to the parental strain (WT) by RAPD profile, and were deemed to be the same strain by this method. Similarities are shown at the tree branches. *B. multivorans* ATCC 17616 is shown as an outlier profile for a different Bcc species, with less than 30% similarity to *B. lata* strain 383 and its preservative-adapted derivatives.

Table 18. Minimum inhibitory concentrations of preservatives for *B. lata* strain 383 and preservative adapted derivatives

B.lata strain	MIC (%) and change (x fold) in MIC from wild-type level													
	MIT/CMIT [1]		MIT		BIT		BC		MP		PH		DH	
wild-type	0.00004681		0.0012125		0.000625		0.0125		0.09375		0.1875		0.0063281	
383-CMIT	0.00018725	x 4	0.0012125	-	0.0025	x 4	0.0125	-	0.09375	-	0.1875	-	0.0063281	-
383-MIT	0.00004681	-	0.0012125	-	0.000625	-	0.00625	x 0.5	0.09375	-	0.1875	-	0.0063281	-
383-BIT	0.00018725	x 4	0.0012125	-	0.0025	x 4	0.00625	x 0.5	0.09375	-	0.1875	-	0.0063281	-
383-BC	0.00004681	-	0.0006063	x 0.5	0.00125	x 2	0.025	x 2	0.046875	x 0.5	0.09375	x 0.5	0.0063281	-

Footnotes:

MIC, minimum inhibitory concentration; MIT/CMIT, methylisothiazolinone and chloromethylisothiazolinone; MIT, methylisothiazolinone; BIT, benzisothiazolinone; BC, benzethonium chloride; MP, methyl paraben; PH, phenoxyethanol; DMH, dimethylol dimethyl hydantoin;

[1] A cosmetic grade commercial blend evaluated

- No change from wild-type level.

% refers to v/v for MIT, MIT/CMIT, PH and DMH; % refers to w/v for other agents.

4.2.2.2 Growth dynamics of adapted *B. lata* derivatives cultured in the absence and presence of preservatives

Growth dynamics of *B. lata* strain 383 and preservative adapted derivatives in the presence and absence of preservatives were determined using a modified microdilution broth assay and a Bioscreen C microbial growth analyser, as described in section 2.13.3. The growth curves generated were used to estimate the length of lag phase and the growth rate (Figure 14 & Figure 15). In the absence of preservatives, the wild-type and derivatives 383-CMIT, 383-MIT and 383-BIT, had a similar lag phase of ~7.5 hours. The adapted derivative 383-BC had a significantly longer lag phase of 10 ± 0.45 hours ($P < 0.05$), in the absence of benzethonium chloride. The mean growth rates of adapted derivatives 383-CMIT and 383-MIT were similar to wild-type levels of ~0.5 generations h^{-1}; however, derivatives 383-BC and 383-BIT were significantly slower ($P<0.05$) at 0.3 ± 0.02 and 0.2 ± 0.02 generations h^{-1}. The length of the lag phase increased and the rate of growth slowed proportionately with increasing concentrations of a preservative agent, for both wild-type and preservative-adapted derivatives. Interestingly, although growth of 383-BC and 383-BIT was slower when cultured in higher concentrations of the agents that had induced resistance (i.e. benzethonium chloride and benzisothiazolinone), the reduction was not significantly different from that observed in preservative-free media (Figure 15, panels D and C).

The highest preservative concentrations, at which the parental strain and adapted derivatives were capable of growth, are shown in Table 19. The isothiazolone-adapted derivative 383-CMIT demonstrated the largest increase in tolerance from wild-type levels: 383-CMIT grew in MIT/CMIT concentrations eight-fold higher than that of the wild-type, after an extended lag phase of 51.2 ± 4.26 hours (Figure 14, panel A). The adapted derivative 383-BIT also demonstrated a large increase in tolerance, and was able to grow in benzisothiazolinone concentrations up to four-fold higher than that of the wild-type, after an extended lag of 36.2 ± 0.3 hours (Figure 14, panel C). Growth curve analysis demonstrated lower levels of adaptive-resistance to methylisothiazolinone and benzethonium chloride preservatives. The BC-adapted strain grew in concentrations of benzethonium chloride that were two-fold higher than that of the wild-type, after an extended lag phase of 30.9 ± 3.4 hours. The growth analysis demonstrated an increased tolerance to methylisothiazolinone in the MIT-adapted derivative that was not evident in earlier evaluations of MIC values: 383-MIT was able to grow in concentrations of MIT two-fold higher than that of the wild-type, after an extended lag of 45 ± 2.2 hours.

The analysis of the growth dynamics of adapted derivatives in the presence of preservatives, suggested the induced mechanisms leading to MIT/CMIT-adaptive resistance conferred cross-resistance to the related isothiazolone compound BIT (Table 19). Growth dynamics did not indicate that preservative-induced adaptive resistance conferred cross-resistance to unrelated compounds. The benzethonium chloride-adapted derivative, which had demonstrated an increase in MIC to benzisothiazolinone (Table 18), failed to grow in concentrations of the agent higher than that of the parental strain (Table 19). In addition, none of the preservative-adapted derivatives grew in medium containing higher concentrations of methyl paraben, phenoxyethanol, and DMDM hydantoin, than that tolerated by the parental strain (Table 19).

Preservative-adapted *B. lata* strain 383 derivatives were incapable of growth in media containing concentrations of preservatives higher than the maximum regulated levels for use in personal care products, in EU-regulated countries (see Table 2 for levels). The growth of the preservative-adapted derivatives required the following fold-reduction from maximum regulated levels: a three-fold reduction of benzisothiazolinone levels for the BIT-adapted derivative; a four-fold reduction of MIT/CMIT and benzethonium chloride enabled the growth of 383-CMIT and 383-BC derivatives; and an eight-fold reduction of methylisothiazolinone was required for the growth of isothiazolone-adapted derivatives.

Table 19. The highest preservative concentration in which *B. lata* strain 383 and adapted derivatives were capable of growth

B.lata strain	Preservative concentration (%) and change (x fold) from wild-type level													
	MIT/CMIT [1]		MIT		BIT		BC		MP		PH		DMH	
wild-type	0.00004683		0.00060625		0.00125		0.0125		0.0938		0.09375		0.00506	
383-CMIT	0.00037462	x 8	0.00121250	x 2	0.00250	x 2	0.0063	x 0.5	0.0938	-	0.09375	-	0.00506	-
383-MIT	0.00004683	-	0.00121250	x 2	0.00250	x 2	0.0125	-	0.0938	-	0.09375	-	0.00506	-
383-BIT	0.00018731	x 4	0.00121250	x 2	0.00500	x 4	0.0125	-	0.0469	x 0.5	0.09375	-	0.00506	-
383-BC	0.00004683	-	0.00060625	-	0.00125	-	0.0250	x 2	0.0469	x 0.5	0.09375	-	0.00506	-

Footnotes:

MIT/CMIT, methylisothiazolinone and chloromethylisothiazolinone; MIT, methylisothiazolinone; BIT, benzisothiazolinone; BC, benzethonium chloride; MP, methyl paraben; PH, phenoxyethanol; DMH, dimethylol dimethyl hydantoin.

[1] A cosmetic grade commercial blend evaluated

- No change from wild-type level.

% refers to v/v for MIT, MIT/CMIT, PH and DMH; % refers to w/v for other agents.

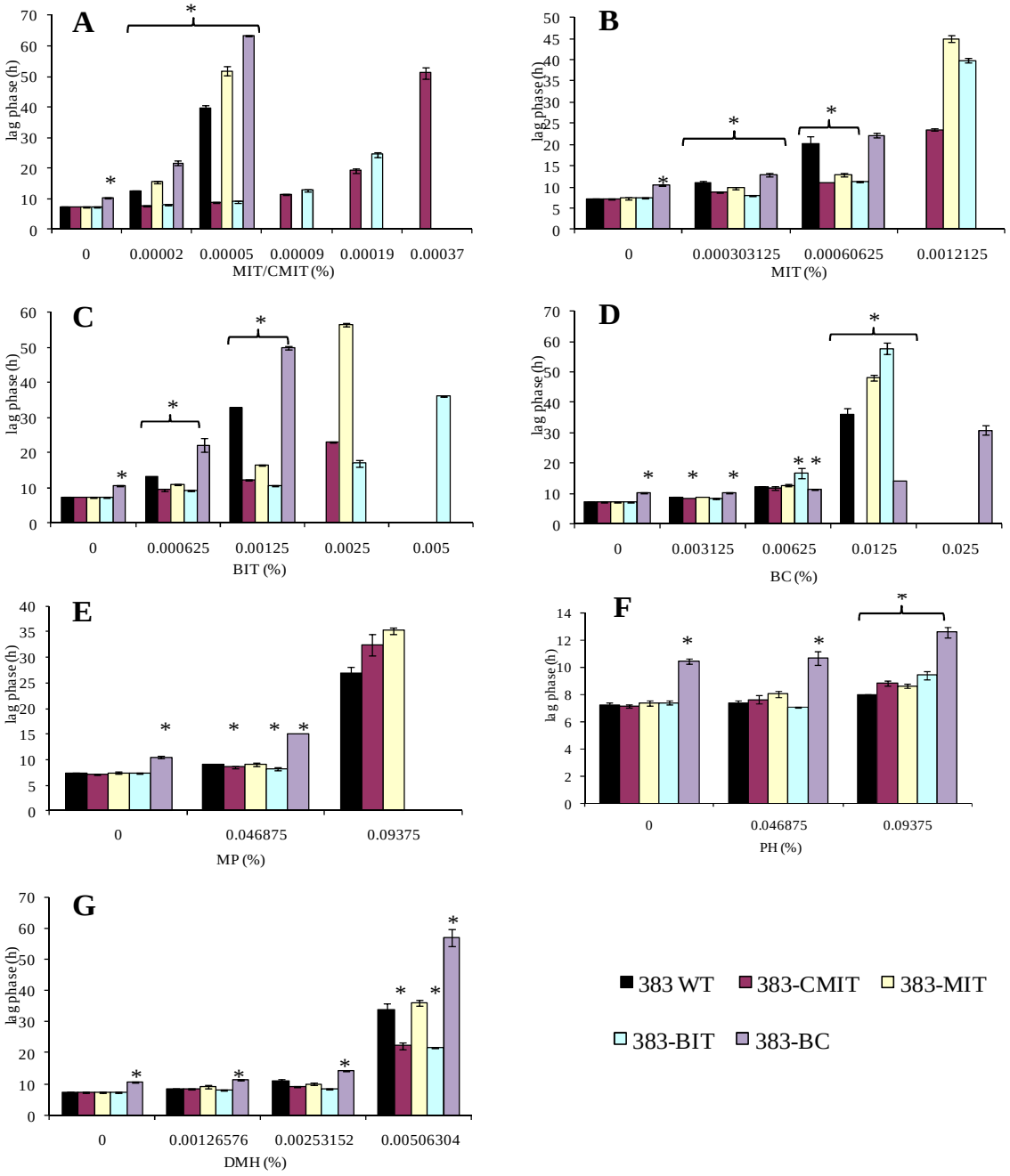


Figure 14. The effect of increasing preservative concentration on the mean lag phase of *B. lata* strain 383 and preservative adapted derivatives.

The mean lag phase (hours) of B. lata strain 383 wild-type (WT) and adapted derivatives cultured in BSM (CYG) broth containing **(A)** MIT/CMIT isothiazolinone blend **(B)** methylisothiazolinone **(C)** benzisothiazolinone **(D)** benzethonium chloride **(E)** methyl paraben **(F)** phenoxyethanol **(G)** DMDM hydantoin preservative agents. All lag phases were over 5 hours, absent bars indicate no growth at test concentration. The parental and derivative strains failed to grow at concentrations higher than that shown in the figure. Error bars show the standard error (SE) of six technical replicates from two biological replicates. Significant differences ($P \leq 0.05$) from the wild-type at individual test conditions are denoted by an asterisk (Kruskal-Wallis and Mann-Whitney (two-tailed) statistical tests).

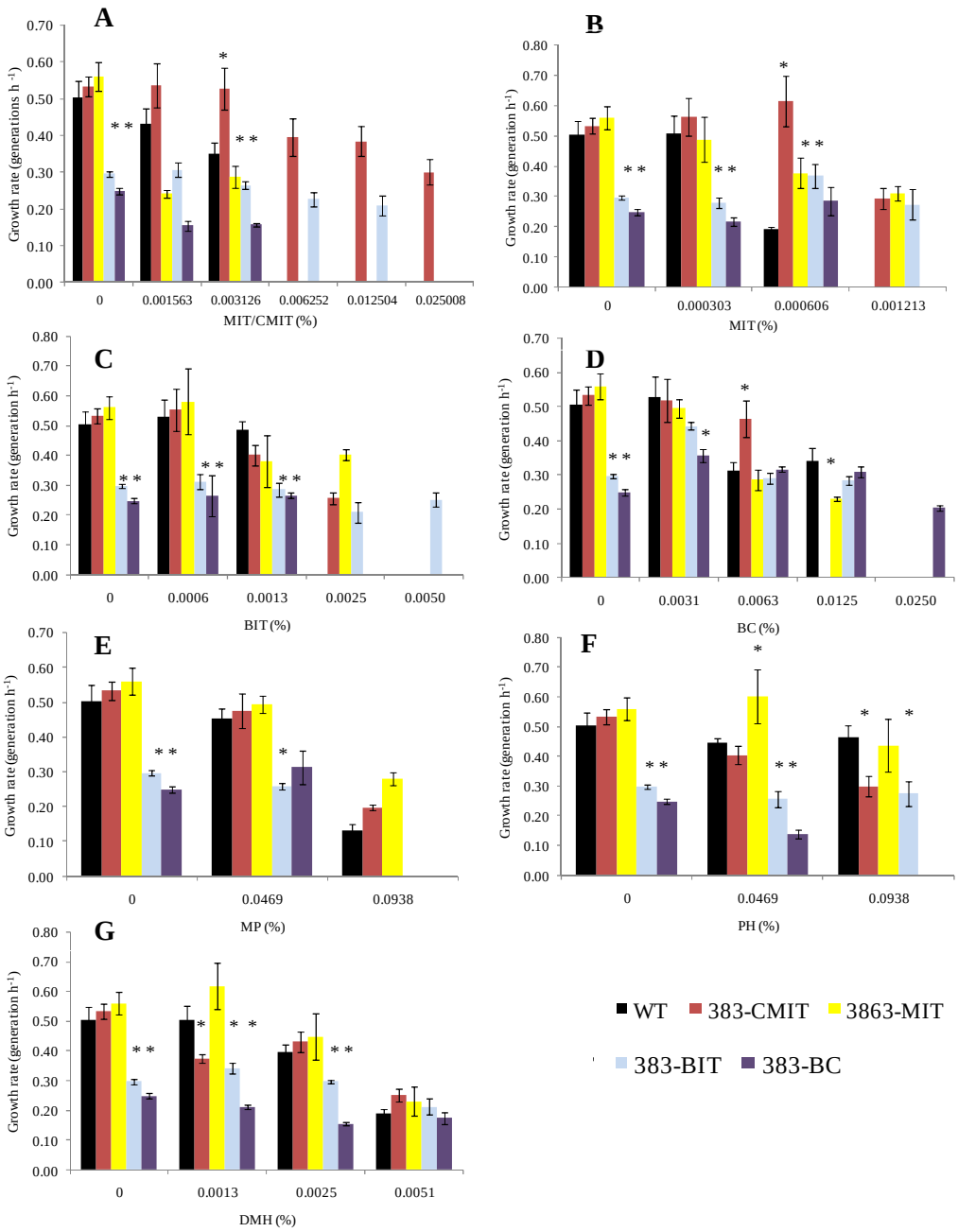


Figure 15. The effect of increasing preservative concentration on the mean growth rate of *B. lata* strain 383 and preservative adapted derivatives.

The mean growth rate (generations h-1) of *B. lata* strain 383 wild-type (WT) and adapted derivatives cultured in BSM (CYG) broth containing (A) MIT/CMIT isothiazolinone blend (B) methylisothiazolinone (C) benzisothiazolinone (D) benzethonium chloride (E) methyl paraben (F) phenoxyethanol (G) DMDM hydantoin preservative agents. The parental and derivative strains failed to grow at concentrations higher than that shown in the figure. Absent bars indicate no growth at the test concentration. Error bars show the standard error (SE) of six technical replicates from two biological replicates. Significant differences ($P \leq 0.05$) from the wild-type at individual test conditions are denoted by an asterisk (Kruskal-Wallis and Mann-Whitney (two-tailed) statistical tests).

4.2.3 Antibiotic susceptibility of adapted *B. lata* derivatives

The susceptibility to eight antibiotics, determined using antibiotic Etest® strips, of *B. lata* strain 383 and preservative-adapted derivatives, is shown in Table 20. The MIC of imipenem and azithromycin for wild-type and adapted derivatives exceeded the highest concentration on the Etest strip; therefore, an increase in tolerance to either agent could not be detected using this method. The MIC values of ceftazidime, trimethoprim/sulfamethoxazole and piperacillin did not vary greater than two-fold from wild-type levels.

Preservative-adapted derivatives 383-CMIT and 383-BIT demonstrated a large (>25-fold) increase in tolerance to ciprofloxacin, increasing from 1.25 to >32 µg/ul (Table 20). These derivatives also demonstrated a >two-fold increase in MIC for chloramphenicol, from 160 to >256 µg/ml - exceeding the highest concentration of the Etest strip (Table 20). In contrast to the increased tolerance to ciprofloxacin, 383-CMIT and 383-BIT derivatives demonstrated an increased susceptibility to amikacin - with an eight-fold and 19-fold reduction in MIC respectively (Table 20). The MIC values of ciprofloxacin and amikacin for the derivative 383-MIT were similar to wild-type levels in contrast to 383-CMIT and 383-BIT. The antibiotic tolerance of the benzethonium chloride-adapted had not increased; rather, *B. lata* 383-BC demonstrated a three-fold decrease in the MIC for amikacin and a two-fold decrease in the MIC for chloramphenicol was detected.

Using an overall antibiotic profile score, as a simplistic measure of multi-drug resistance in the preservative adapted derivatives (Rose *et al.*, 2009), all derivatives had a profile score lower than the parental strain. *B. lata* strain 383 (wild-type) had the highest score of >84.69, and derivative 383-BC had the lowest score of >58.20.

Table 20. Antibiotic MIC for *B. lata* strain 383, the preservative-adapted derivatives and reference organisms

Organism	Mean MIC of strains (µg/ml)								Antibiotic profile score [1]
	AMK	AZM	CAZ	CIP	CHL	IMP	PIP	SXT	
***B. lata* strain**									
383 wild-type	192	>256	12	1.25	160	>32	24	0.25	>84.69
383-MIT	128	>256	10	1.5	128	>32	24	0.25	>72.47
383-CMIT	24	>256	12	>32	>256	>32	24	0.315	>79.54
383-BIT	10	>256	10	>32	>256	>32	16	0.25	>76.53
383-BC	64	>256	12	1.25	72	>32	28	0.315	>58.20
Reference strain									
S. aureus NCTC 12981	3	3 [α]	32	0.38	6	0.25	4	0.125	6.54
E.coli NCTC 12241	2.5	16	1.5	0.094	8	1 [α]	28 [α]	0.19	4.71
P. aeruginosa NCTC 12903	2.5	>256	7	0.25	>256	>32 [α]	24 [α]	3	>72.59

Footnotes:

MIC, minimum inhibitory concentration; AMK, amikacin; AZM, azithromycin; CAZ, ceftazidime; CIP, ciprofloxacin ; CHL, chloramphenicol ; IMP, imipenem; PIP, piperacillin; SXT, Trimethoprim/sulfamethoxazole. [1] The antibiotic profile score was an average of MIC values used as a measure of overall multi drug resistance. [α] Outside manufacturers quality control range.

4.2.3.1 Fluoroquinolone susceptibility of adapted *B. lata* derivatives

A modified microdilution broth assay was used to investigate cross-resistance to (second and third generation) fluoroquinolone antibiotics in isothiazolone-adapted derivatives *B. lata* 383-CMIT and 383-BIT. The MIC and MBC of ciprofloxacin, levofloxacin, norfloxacin and sparfloxacin antibiotics for the parental *B. lata* strain and derivatives are shown in Figure 16. Derivatives 383-CMIT and 383-BIT had higher MIC and MBC values than the wild-type and other adapted derivatives for all fluoroquinolone antibiotics. 383-BIT demonstrated a higher tolerance to ciprofloxacin, levofloxacin and sparfloxacin than derivative 383-CMIT. The MIC and MBC values for fluoroquinolone antibiotics for the derivative 383-MIT were closer to wild-type levels (Figure 16). Derivative 383-BC demonstrated higher tolerance to killing by ciprofloxacin and norfloxacin but showed increased susceptibility to sparfloxacin and levofloxacin from wild-type levels (Figure 16). Norfloxacin inhibited growth of all preservative adapted derivatives and the wild-type at ≤256 µg/ml; however, bactericidal concentrations for derivatives 383-CMIT, 383-BIT and 383-BC were found to be four-fold higher than that of the MIC at 1024 µg/ml.

4.2.3.1.1 Screening for mutations in the topoisomerase genes of preservative-adapted derivatives with fluoroquinolone cross-resistance

Resistance to fluoroquinolone antibiotics may arise via the accumulation of mutations in the topoisomerase genes (i.e. *gyrA*, *gyrB*, *parC* and *parE),* which are the primary target of this antibiotic. To determine whether the increased fluoroquinolone resistance of the preservative-adapted derivatives was a result of mutation(s), fragments (≤595 bp) of the topoisomerase genes, that encompassed the following quinolone resistance determining region (QRDR) of each gene, were amplified and then sequenced in both directions: for *gyrA*, the coding region of protein residues 50 – 120 was sequenced, encompassing the putative QRDR of protein residues 67 – 106 (Hopkins *et al.*, 2005; Pope *et al.*, 2008); for *gyrB* , the coding region of protein residues 376 – 520 was sequenced , encompassing the putative QRDR of protein residues 426 – 447 (Hopkins *et al.*, 2005) ; for *parC*, the coding region of protein residues 50 – 100 was sequenced, encompassing the putative QRDR of protein residues near Ser80 (Pope *et al.*, 2008); for *parE*, the coding region of protein residues 400 – 600 was sequenced, encompassing the putative QRDR of protein residues 420 – 529 (Hopkins *et al.*, 2005). On comparison, the aligned consensus sequences were found to be identical (appendix 9.1); the preservative-adapted derivatives had not acquired mutations within or outside the putative QRDR of the topoisomerase genes.

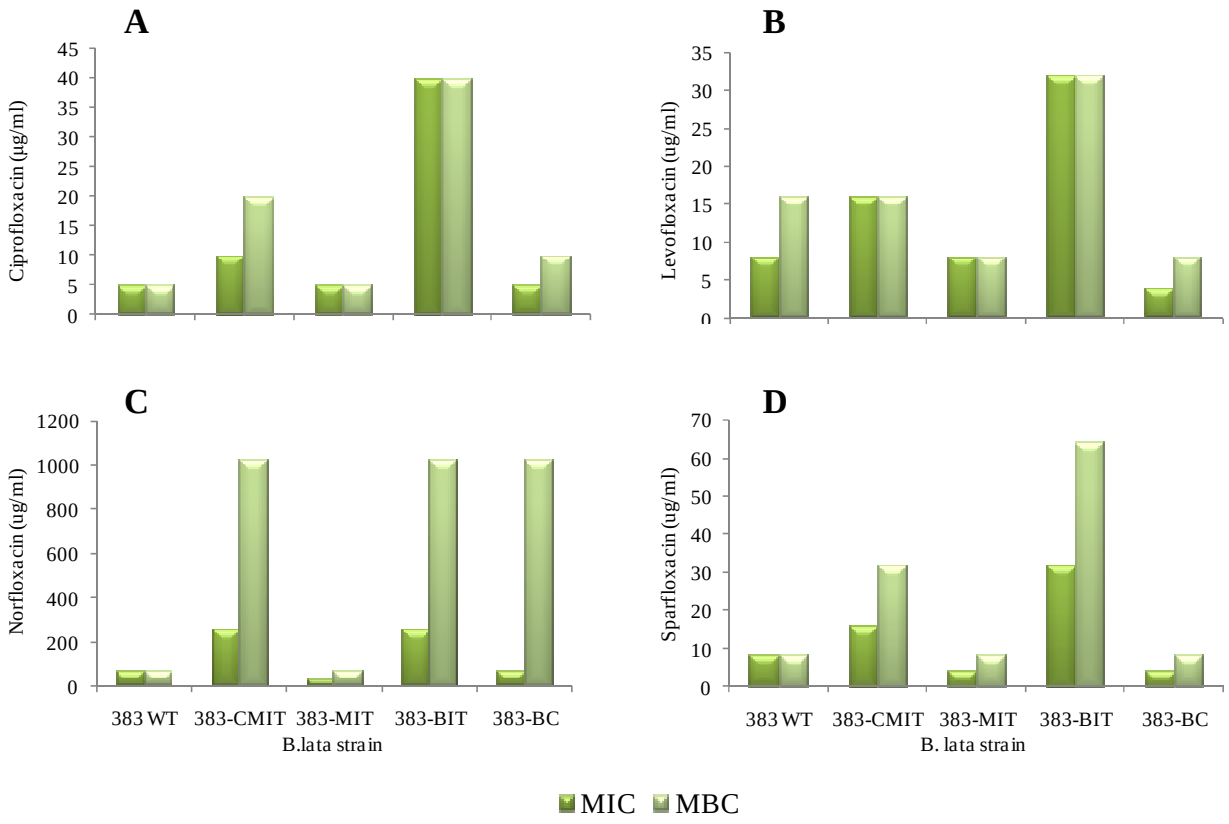


Figure 16. Susceptibility of *B. lata* strain 383 and preservative adapted derivatives to fluoroquinolone antibiotics.

The minimum inhibitory and minimum bactericidal concentrations of (**A**) ciprofloxacin (**B**) levofloxacin (**C**) norfloxacin (**D**) sparfloxacin fluoroquinolone antibiotics, determined using a modified broth microdilution assay based on CLSI guidelines (published 2006). The MIC and MBC derived from four technical replicates within one biological replicate are shown.

4.2.4 Assessing the phenotypic stability of preservative-induced adaptive resistance of *B. lata* strain 383

To assess the stability of preservative-induced adaptive resistance of *B. lata* strain 383, adapted-derivatives were repeatedly sub-cultured in the absence of the priming preservative. RAPD analysis was used to confirm the identity *B. lata* strain 383 preservative-adapted derivatives after sub-culture, as had been applied to the original adapted derivatives after isolation (section 4.2.1). After five and ten subcultures, the growth dynamics of the adapted-derivatives in media containing a range of preservative concentrations, and the antibiotic susceptibility were re-assessed. Within ten sub-cultures, the preservative and antibiotic susceptibility (Tables 18 and 20) of the preservative-adapted derivatives (383-CMIT, 383-MIT, 383-BIT and 383-BC) did not revert to wild-type levels.

4.2.5 Biofilm formation of preservative-adapted *B. lata* strain 383 derivatives

The formation of biofilm biomass by preservative-adapted derivatives, cultured in the absence and presence of sub-inhibitory preservative concentrations, was determined using a crystal-violet staining assay. The biofilm formation of the derivatives 383-CMIT, 383-MIT and 383-BIT was similar to that of the parental *B. lata* strain 383 (Figure 17). The benzethonium chloride-adapted derivative produced significantly more ($P < 0.001$) biofilm biomass than that of the parental strain in BSM (CYG) broth, in the absence and presence of preservatives other than benzethonium chloride. An exception to this biofilm positive phenotype was that, when cultured in the presence of sub-inhibitory concentrations of benzethonium chloride, the amount of biofilm biomass formed by the derivative 383-BC, reduced to that of parental strain (Figure 17, panel D). In addition, this derivative tolerated, and formed a biofilm biomass in, BSM (CYG) broth at pH 5, in the presence and absence of sodium benzoate. Under these conditions, the parental strain and isothiazolone-adapted derivatives did not form a biofilm biomass that was detectable by this method (Figure 17, panel G).

Biofilm formation of the preservative-adapted derivatives, in response to sub-inhibitory preservative concentrations, did not significantly increase ($P > 0.05$) (Figure 17). Although not significant ($P > 0.05$), biofilm formation of the adapted-derivative 383-BC increased in response to benzisothiazolinone and methyl paraben (Figure 17, panels D and F); biofilm formation of 383-BIT also increased in response to sub-inhibitory concentrations of phenoxyethanol (Figure 17, panel H).

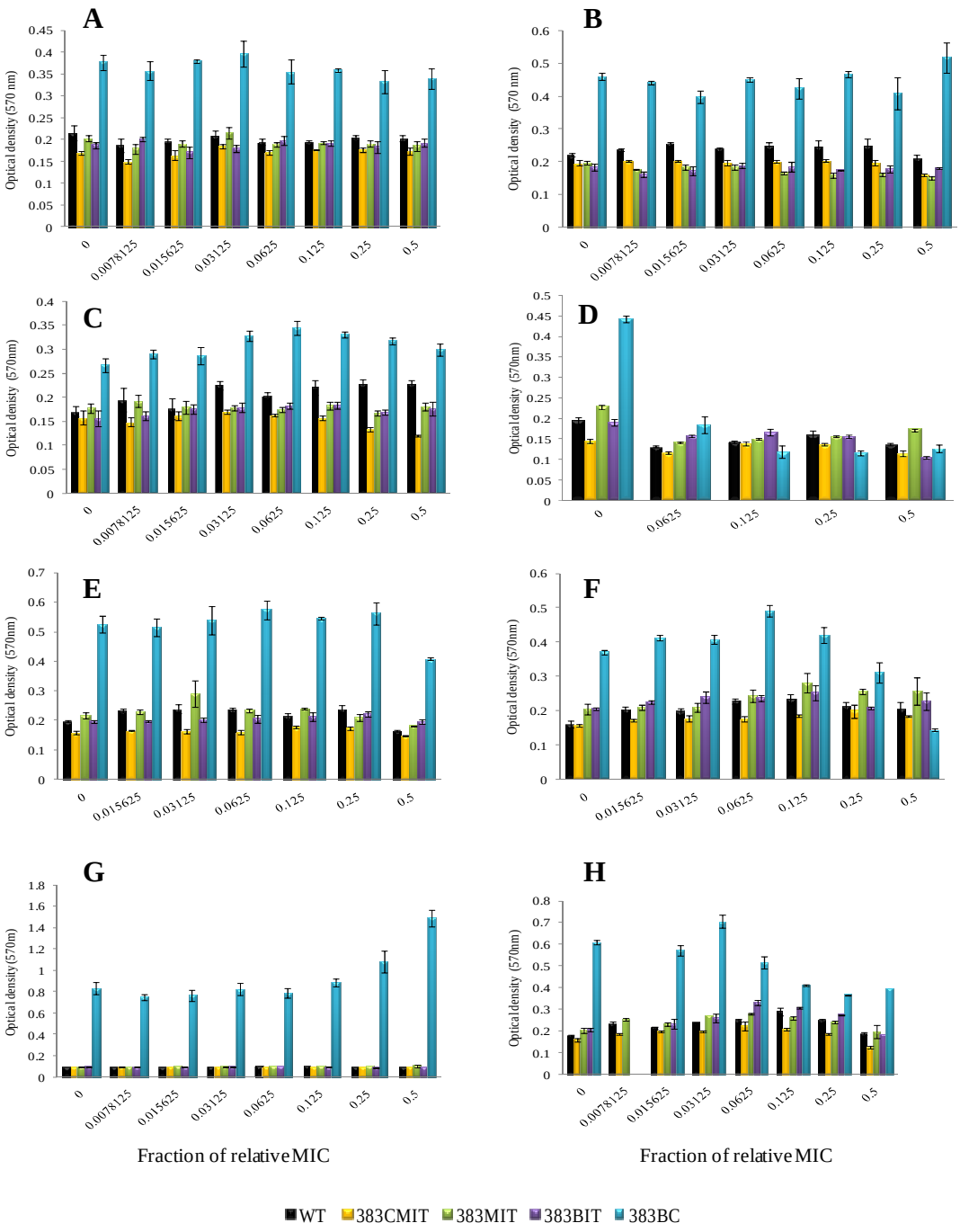


Figure 17. The effect of sub-inhibitory preservative concentrations on the biofilm biomass of *B. lata* strain 383 and preservative adapted derivatives.

The mean absorbance, obtained by crystal violet assay, of biofilms of *B. lata* strain 383 and preservative adapted derivatives 383-CMIT, 383-MIT, 383-BIT and 383-BC cultured in BSM (CYG) broth containing sub-inhibitory concentrations of **(A)** MIT/CMIT isothiazolinone blend **(B)** methylisothiazolinone **(C)** benzisothiazolinone **(D)** benzethonium chloride **(E)** DMDM hydantoin **(F)** methyl paraben **(G)** sodium benzoate **(H)** phenoxyethanol. Preservative concentrations are presented as fractions of the relative MICs. Sodium benzoate was assayed at pH 5, all other comparisons were conducted at pH 7. Error bars indicate standard error of the mean of triplicate stained wells of a test plate. Kruskal-Wallis and Mann-Whitney (one-tailed) statistical tests revealed there was no significant increase in biofilm production from control levels, in the presence of sub-inhibitory concentrations of the preservatives ($P>0.05$). 383-BC and 383-CMIT had significantly different ($P<0.002$) biofilm production from the wild-type in control conditions.

4.2.6 Swarming motility of adapted *B. lata* derivatives

The swarming motility of preservative-adapted derivatives of *B. lata* strain 383, on semi-solid (preservative free) swarm plates, is shown in Figure 18. Swarming motility of the adapted derivative 383-MIT was similar to the wild-type: it exhibited the characteristic blue-green pigmented dendritic swarm, and reached a diameter of 50.5 ± 0.3mm at 48 hours (Figure 18, panel D). The adapted derivatives 383-CMIT and 383-BIT demonstrated reduced swarming motility: the diameter of the swarms reached a mean diameter of 27.5 ± 1.1 mm and 27.5 ± 0.3 mm (Figure 18, panels C and E). In addition, the adapted derivatives 383-CMIT and 383-BIT displayed an altered morphology and reduced pigmentation, as shown in Figure 18, panels C and E. The adapted derivative 383-BC also displayed poor swarming motility, with a swarm diameter of 26 ± 1.4 mm; however, the swarm morphology and pigmentation were similar to that of the parental strain (Figure 18, panel F).

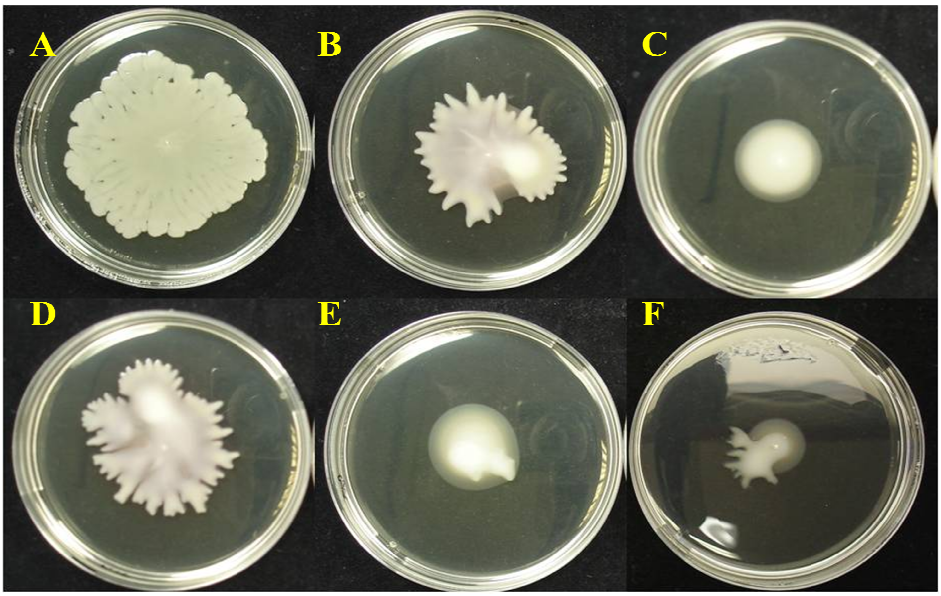


Figure 18. Swarming motility of *B. multivorans* ATCC 17616, *B. lata* strain 383 and preservative-adapted derivatives of *B. lata* strain 383.

The swarm morphology of **(A)** *B. multivorans* ATCC 17616 **(B)** *B. lata* strain 383 wild-type **(C)** *B. lata* 383-CMIT **(D)** *B. lata* 383-MIT **(E)** *B. lata* 383-BIT **(F)** *B. lata* 383-BC on semi-solid isosensitest swarm agar (without preservatives) at 48 hours. The mean swarm diameter (± STDEV) for the respective strains was as follows: 55.5 ± 6.5 mm, 50 mm, 27.5 ± 1.1 mm, 50.5 ± 0.3 mm, 27.5 ± 0.3 mm and 26 ± 1.4 mm.

4.3 DISCUSSION

4.3.1 Stable adaptive resistance to preservatives can be developed in *B. lata* strain 383

The preservative-induced adaptive resistance of *B. lata* strain 383 was investigated via the progressive sub-culture of the strain on media containing increasing sub-inhibitory concentrations of an individual preservative. This approach represented exposure to low levels of an active ingredient that may occur as a result of the inadequate sanitation of production lines (i.e. residue of a dilute industrial product); the leaching or binding of a preservative or preservatives within a system; and/or the deleterious activity of formulation excipients on a preservative system. The investigation of Bcc adaptation to combinations of preservatives, often used in industry, and product formulation (that may possess an intrinsic anti-Bcc activity (Maillard, 2009), was beyond the scope of this study.

Adaptive resistance was developed in *B. lata* strain 383 to methylisothiazolinone, the MIT/CMIT isothiazolinone blend, benzisothiazolinone, and benzethonium chloride preservatives: these agents were from two of the five classes of preservatives evaluated in this study. Preservative-induced adaptive resistance was remarkably stable. The observed increase in the level of resistance persisted in the absence of the priming agent, without reversion to wild-type levels. This suggests that preservative-induced resistance in *B. lata* strain 383 may not be transient in nature, and that resistance, once developed, may persist.

A high degree of stable adaptation to the blend of MIT/CMIT isothiazolone agents was observed in *B. lata* strain 383. Exposure to sub-inhibitory concentrations of the isothiazolones induced resistance mechanisms that resulted in a four-fold increase in the MIC, and the ability to grow in the presence of MIT/CMIT concentrations eight-fold higher than that of the parental strain. High level adaptation to the (cosmetics grade) isothiazolone blend has also been documented in other bacterial species. For example, Orth and Lutes (Orth & Lutes, 1985) reported adaptation to MIT/CMIT, in which *S. aureus, P. aeruginosa* and *E.coli* increased their tolerance by 5 -, 10-, and 30-fold respectively. Interestingly, the investigation of isothiazolone-induced adaptive resistance in *P. aeruginosa* implies that the chemical form of preservatives may also affect the stability of preservative-induced adaptation. Isothiazolinone-induced resistance in *P. aeruginosa* has been accompanied by the loss of a 35-kDa outer membrane protein (T-OMP), presumed to be involved in the uptake of isothiazolone molecules (Brozel &

Cloete, 1994). Following exposure to the cosmetics-grade blend of isothiazolone preservatives (MIT/CMIT), levels of resistance, and the outer membrane profiles of the adapted-derivative, did not revert to wild-type levels when cultured in the absence of the priming agents (Brozel & Cloete, 1994). In contrast, levels of adaptive-resistance induced by the isothiazolones in their purest chemical forms, were less persistent. In the absence of the priming preservatives, levels of resistance demonstrated a reversion to wild-type levels, and in association, the T-OMP reappeared in the outer membrane profile (Winder *et al.*, 2000). The effect of preservative blends, in contrast with chemicals in their purest form, on the stability of Bcc adaptation, may be worthy of further investigation.

The cost to fitness (i.e. the rate of growth) of preservative-induced adaptive resistance in *B. lata* strain 383, appeared to be agent specific. Adaptation to methylisothiazolinone and the blend of MIT/CMIT was less costly to fitness, as it did not significantly alter the growth rate or length of lag-phase. Benzisothiazolinone and benzethonium chloride induced adaptive resistance reduced the fitness of the derivatives, this was indicated by a slower growth rate and lag-phase increased to that of the wild-type. These are characteristics commonly associated with bacterial adaptation to antimicrobials (Joynson *et al.*, 2002). Interestingly, the extended lag-phase of the BC-adapted derivative significantly reduced in the presence of sub-inhibitory concentrations of the priming preservative (BC). This suggests that following adaptation to benzethonium chloride, the agent is required for normal growth characteristics.

The survey of Bcc susceptibility to preservatives used in industry (discussed in Chapter 3) revealed that Bcc isolates from environmental-industrial sources were significantly more tolerant to DMDM hydantoin than Bcc from clinical and environmental sources. Interestingly, DMDM hydantoin-induced adaptive resistance in *B. lata* strain 383 was not promoted, via progressive sub-culture on BSM agar containing sub-inhibitory concentrations; and cross-resistance to DMDM hydantoin was not observed in any of the four preservative-adapted derivatives. Similarly, adaptive resistance to methyl paraben and phenoxyethanol was not promoted by this method, and the four preservative-adapted derivatives did not display cross-resistance to these agents. Adaptive-resistance to DMDM hydantoin, methyl paraben and phenoxyethanol, has been successfully induced via progressive culture in media containing sub-inhibitory concentrations, in other bacterial species including *P. aeruginosa, E. coli* and *S. aureus* (Abdel Malek & Badran, 2010; Orth & Lutes, 1985). This suggests that these agents may be recalcitrant to *B. lata* strain 383

adaptation. Whether adaptive resistance plays an important role in the selection of Bcc with elevated tolerance to DMDM hydantoin in the environmental-industrial niche remains to be determined. In the survey of Bcc preservative susceptibility (Chapter 3), strains demonstrating the highest MICs for DMDM hydantoin belonged to *B. arboris*, *B. cenocepacia* and *B. multivorans* species groups. Bcc adaptation to DMDM hydantoin methyl paraben and phenoxyethanol, may be species or even strain specific, and/or require environmental factors not replicated in this study.

Whether preservative-induced Bcc adaptive-resistance facilitates adaptation to other antimicrobials is also worthy of further investigation. The competence of the preservative-adapted *B. lata* derivatives to develop additional adaptive-resistance to preservative agents such as DMDM hydantoin, methyl paraben and phenoxyethanol, or to biocides used as disinfectants, was not assessed in this study.

4.3.2 Preservative cross-resistance of *B. lata* 383 derivatives

Isothiazolone-adapted derivatives 383-CMIT and 383-BIT demonstrated cross-resistance to benzisothiazolone and the blend of MIT/CMIT preservatives; these are related compounds that are considered to have a similar mode of action and cellular target (Collier *et al.*, 1990a; Denyer, 1995). The preservative susceptibility profile of the isothiazolone-adapted derivatives varied, and adaptation to methylisothiazolinone did not confer cross-resistance to other agents of the same class. This suggests that the mechanism or combination of mechanisms leading to isothiazolone-adaptive resistance may be agent specific. Adaptation to benzethonium chloride was not associated with a reproducible increase of tolerance to the unrelated preservative agents evaluated, and cross-resistance to related QACs was not investigated.

Cross-resistance to additional biocides, used as disinfectants and/or antiseptic agents, was not evaluated in this study. This is worthy of investigation as cross-resistance among preservatives and disinfectants has potentially serious implications in the ability to eradicate resistant organisms from contaminated surfaces, or manufacturing processes, using disinfectants (Chapman, 1998). Many classes of preservative agents, such as alcohols, QAC, formaldehyde, bisguanides, and bisphenols, also function as disinfectants and/or antiseptics at higher concentrations (Russell, 2004b). Although the reduction in susceptibility, associated with preservative-induced adaptive resistance, is generally quite low, any reduction in susceptibility can be a problem (Chapman, 1998); whether

preservative-induced adaptive resistance contributes to the selection and breakthrough of biocide resistant Bcc bacteria remains to be explored.

4.3.3 Adaptation to preservatives can confer stable cross-resistance to antibiotics

In recent years, there have been growing concerns that the extensive use of antimicrobials, in domestic and industrial settings, may be a contributing factor to the development and selection of antibiotic resistant strains (Gilbert & McBain, 2003). The present study demonstrated that although the mechanisms leading to preservative-induced adaptive resistance in *B. lata* strain 383 may not confer multi-drug resistance, they may be associated with a stable reduction in susceptibility to clinically relevant antibiotics such as the fluoroquinolones.

Fluoroquinolone antibiotics have dual targets, topoisomerase II (DNA gyrase) and topoisomerase IV, which are related but distinct enzymes involved in DNA synthesis (Chen & Lo, 2003). Two classically described resistance mechanisms have been associated with fluoroquinolone resistance in Bcc bacteria:

(1) an accumulation of mutations in quinolone resistance determining region (QRDR) of the topoisomerase genes, resulting in amino acid substitutions, that can weaken quinolone binding;

(2) a reduction of the intracellular accumulation of the drug by active efflux (Pope *et al.*, 2008).

Sequence analysis of the *B. lata* strain 383 derivatives revealed that fluoroquinolone resistance was not mediated by target modification; resistance was mediated by a different mechanism or combination of mechanisms such as active efflux and/or decreased permeability. The observed levels of reduced susceptibility to isothiazolinone preservatives, and fluoroquinolone antibiotics (a <100 fold increase in MIC for both agents), are indicative of active efflux as appose to target modification, and/or enzymatic degradation of the drug (Piddock, 2006).

Interestingly, adaptive resistance to isothiazolinone agents (CMIT, BIT) conferred increased susceptibility to amikacin. Bcc bacteria are intrinsically resistant to aminoglycoside antibiotics; this inherent trait has been attributed to the structure of the LPS in the outer membrane, and antibiotic modifying enzymes (Coenye, 2007b). The primary target of aminoglycosides is the inhibition of ribosomal protein synthesis. To enter the cell, cationic compounds such as amikacin use a self-promoted uptake system

involving the interaction of polycation with the divalent cation-binding site of the LPS (Coenye, 2007b). Binding of the aminoglycoside results in the disruption of the outer membrane, and increases uptake of the agent. The structure of the Bcc LPS conceals the iron cationic binding sites, while the unique composition of the core oligosaccharide, which contains less phosphate and 3-deoxy-D-manno-octo-2-ulsonic acid than the LPS of other Gram negative bacteria, neutralise the negative charge to the outer membrane (Coenye & Vandamme, 2007b). Aminoglycoside-inactivating enzymes, which may modify a specific amino or hydroxyl group of the compound, contribute to the intrinsic resistance of Bcc bacteria, but are unlikely to serve as the sole resistance mechanism (Coenye & Vandamme, 2007b). An increased susceptibility to amikacin, in isothiazolinone-adapted *B. lata* derivatives 383-CMIT and 383-BIT, may be an indication of changes to the LPS, the outer membrane, and/or the reduced activity of chromosomally-encoded aminoglycoside-inactivating enzymes.

The variation in antibiotic susceptibility between the isothiazolone and benzethonium chloride-adapted *B. lata* 383 derivatives, provides further evidence that the induced mechanisms of adaptive-resistance are agent-specific. Further investigation of antibiotic cross-resistance in other preservative-adapted Bcc strains may ascertain whether preservative-induced mechanisms are also strain specific.

4.3.4 Adaptive resistance to preservatives can alter biofilm formation and swarming motility

The effect of preservative-induced adaptive resistance on the swarming motility, pigment production, and biofilm formation of *B. lata* strain 383 appeared to be agent specific: adaptation to methyliosthiazolione was not associated with a significant alteration to these traits; while the isothiazolone-adapted derivatives 383-CMIT and 383-BIT demonstrated a reduced swarming motility. Adaptation to benzethonium chloride was associated with a reduction in swarming, an increase of pigment production, and an increase of biofilm formation.

The association of preservative-induced adaptive resistance and an increase of biofilm formation is a trait that may have serious implications in an industrial setting; as bacteria associated within biofilms are considered to be significantly less susceptible to preservatives and biocides than their planktonic equivalent (Cloete, 2003; Donlan & Costerton, 2002; Morton *et al.*, 1998). Combined with the inherent resistance of the

bacterial biofilm, low levels of preservative-induced adaptive resistance may be amplified. Additionally, the limited penetration of antimicrobials through the biofilm may facilitate the further adaptation of sheltered cells and the selection of resistant organisms (Chapman, 2003a; Szomolay *et al.*, 2005).

Quorum-sensing systems have been associated with the regulation of an arsenal of Bcc genes, including those involved in swarming motility, biofilm formation and pigment production (Eberl, 2006; Venturi *et al.*, 2004). The altered phenotypic traits of adapted *B. lata* strain 383 derivatives suggest that preservative-induced resistance mechanisms may be associated with an alteration in the secretion, sensing and/or regulation of QS systems. Wopperer *et al.* (2006) demonstrated the degradation of short- and long-acyl-chain homoserine lactone (AHL) signalling molecules, utilised by Bcc bacteria, abolished swarming motility, and significantly reduced pigment production. The role of AHLs in biofilm formation has been shown to be strain-specific, decreasing biofilm formation in many Bcc strains but increasing the biofilm biomass of others. The putative role of quorum sensing in the preservative-induced adaptive resistance of Bcc bacteria remains to be examined.

4.3.5 Adaptive resistance and preservative challenge testing

Preservative challenge or efficacy tests are used to determine whether a raw material and/or finished product, are adequately preserved and therefore hygienically secure. Test samples are challenged with a single or mixed inoculum of recommended test organisms that represent organisms likely to be encountered as contaminants (Russell, 2003a). Reference microorganisms from culture collections are typically recommended for use in challenge tests, and although not recommended by all efficacy test methods, manufacturers generally include adapted ‘in-house’ isolates that have been recovered from the manufacturing process (Orth *et al.* 2006)).

Reference strains represent the genetic diversity of encountered contaminants but they may fail to evaluate the efficacy of a preservative system against native adapted organisms, which may possess resistance traits (i.e. intrinsic, acquired and adaptive) shaped by the selective pressures of an industrial environment. This study demonstrated a clear distinction in the susceptibility, and additional phenotypic traits, of a reference strain (*B. lata* strain 383) and its preservative-adapted derivatives. Preservative efficacy tests

utilising only un-adapted reference strains, increase the risk of overestimating preservative efficacy, and as a result may increase the likelihood of product failure.

Preservative-induced adaptive resistance of *B. lata* strain 383 persisted in the absence of the priming agent: resistance did not revert to wild-type levels. This suggests preservative-induced adaptive resistance has the potential to contribute to the development of Bcc resistance to preservatives, as any reduction in susceptibility may favour the subsequent acquisition of additional resistance traits (via mutation or horizontal gene transfer from other resistant species) that may lead to high level resistance, and the breakthrough of resistant organisms (Fernández *et al.*, 2011). Challenge tests are designed to evaluate the efficacy of preservatives in protecting a product or material from microbial insult, but they do not consider the capacity of an agent, or combinations of preservatives, to induce bacterial adaptation that may lead to a reduction in susceptibility. The potential for the induction of adaptive-resistance should perhaps be assessed in parallel to the ability of a preservative agent or system to inhibit and/or kill challenge test organisms.

4.4 CONCLUSIONS

The main conclusions gained from the study of preservative-induced adaptive resistance in Bcc are:

1) The progressive sub-culture of *B. lata* strain 383 on increasing sub-inhibitory concentrations of isothiazolone and benzethonium chloride preservatives developed stable (not transient) adaptive resistance. The greatest reduction in susceptibility was associated with the cosmetic grade blend of MIT/CMIT preservatives. Further investigation is required to establish whether the mechanisms leading to preservative-induced adaptive resistance, the level of reduced susceptibility, and the stability of adaptation are Bcc species and/or strain-specific.

2) Preservative-adapted *B. lata* strain 383 derivatives remained susceptible to levels of preservatives regulated for use in personal care products.

3) Adaptive resistance to DMDM hydantoin, phenoxyethanol and methyl paraben preservatives could not be promoted in *B. lata* strain 383 by progressive subculture on agar containing sub-inhibitory preservative concentrations. Adaptive resistance to these agents may be Bcc species and/or strain specific, or require environmental factors not replicated in this study.

4) Growth curve analysis suggested that the fitness cost of preservative-induced adaptive resistance to *B. lata* strain 383, may be agent specific: adapted derivatives 383-BIT and 383-BC had slower growth rates than other adapted derivatives, and the parental strain. Following adaptation to benzethonium chloride, the *B. lata* strain 383-BC derivative required the presence of the preservative for normal growth.

5) The preservative and antibiotic susceptibility profiles of preservative-adapted *B. lata* strain 383 derivatives varied. This suggested that the mechanisms leading to preservative-induced adaptive resistance may be agent-specific.

6) The mechanisms leading to MIT/CMIT and BIT-induced resistance conferred stable cross-resistance to fluoroquinolone antibiotics. Fluoroquinolone resistance, observed in adapted derivatives 383-CMIT and 383-BIT, was not mediated via target modification; therefore, resistance was likely to be mediated by a non-specific resistance mechanism or combination of mechanisms, such as active efflux and/or decreased cellular permeability.

7) Overall, preservative-induced adaptive resistance in *B. lata* strain 383 was not associated with multi-drug resistance: the preservative-adapted derivatives had an antibiotic susceptibility profile lower than that of the parental strain.

8) Preservative-induced adaptive resistance in *B. lata* strain 383 affected swarming motility, pigment production, and biofilm formation, in an agent-specific manner. The alteration of these QS-regulated phenotypic traits may indicate an alteration in the secretion, sensing and/or regulation of quorum sensing systems.

9) The progressive sub-culture of *B. lata* strain 383 in sub-inhibitory concentrations of preservatives did not promote high levels of resistance; preservative-adapted derivatives were susceptible to the maximum levels of preservatives regulated for use in personal care product.

10) A greater understanding of the potential for a preservative agent, or combination of agents, to induce adaptive resistance in Bcc bacteria, may facilitate the design and implementation of improved preservative strategies that resist adaptation, thereby, minimising the risk of developing preservative resistance.

5 THE GENETIC DETERMINANTS FOR PRESERVATIVE RESISTANCE

5.1 INTRODUCTION

Bacterial contamination of preserved industrial products can lead to economic loss, and depending on the contaminating organism, may pose a health risk to vulnerable consumers. *Burkholderia cepacia* complex bacteria, a group of closely related opportunistic pathogens that are ubiquitous in the natural environment, are predominant Gram-negative contaminants of sterile and non-sterile preserved pharmaceuticals and personal care products (Jimenez, 2007). Members of the Bcc are renowned for high levels of intrinsic antimicrobial resistance, and their ability to contaminate antimicrobial solutions, and withstand antibiotic therapy, is well documented (Mahenthiralingam *et al.*, 2008). In order to better target these organisms it is important to understand the molecular and genetic determinants for antimicrobial resistance. In recent decades there have been great advances in the characterisation of Bcc antibiotic resistance mechanisms (Burns, 2007); however, these mechanisms only represent a small minority of the potential resistance determinants of these organisms. The genetic basis for Bcc preservative resistance has not been extensively characterised.

Transposon mutagenesis remains one of the most extensively used genetic techniques for the characterisation of bacteria (Dennis & Zylstra, 1998); chromosomal mutants can either be generated randomly or site-specifically (Schweizer, 2008). There are many well characterised transposons available for the mutagenesis of Gram-negative bacteria (De Lorenzo *et al.*, 1990; de Lorenzo & Timmis, 1994; Hayes, 2003). However, inherent difficulties with their use often necessitate engineering to improve transposition and selection efficiency, as well as versatility and host range.

The problems associated with the use of transposons such as Tn5, have been largely overcome with the development of mini-transposons (Schweizer, 2008; Dennis & Zylstra, 1998). These small specialised transposons, engineered so that the cognate transposase is located outside the transposon's inverted repeats, stably integrate into the target DNA without its transposase; thereby preventing subsequent transpositional events and genomic rearrangements (De Lorenzo *et al.*, 1990). Dennis *et al.* (1998) modified the basic Tn5–based mini-transposon to include: a conditional origin of replication, thereby facilitating the rapid cloning of DNA flanking the site of insertion; exchangeable antibiotic resistance selective markers; and rare endonuclease sites, in order to facilitate the localisation of the

insertion within a physical genomic map by pulse-field gel electrophoresis. The basic construct of the Tn5*Mod* plasposon, with a trimethoprim resistance cassette, is shown in Figure 19. Once introduced into the host bacterium, the selection marker is lost from the cell population unless a transposition event takes place that involves the expression of the transposase gene, and the stable integration of the marker gene into the host genome (Leveau *et al.*, 2006). Since its original publication (Dennis & Zylstra, 1998), plasposon technology has been successfully used to generate mutant libraries of several α-, β- and γ-proteobacteria; and has been successfully applied to the study of Bcc bacteria (Dennis & Zylstra, 1998; Engledow *et al.*, 2004; Fehlner-Gardiner *et al.*, 2002; Fehlner-Gardiner & Valvano, 2002; Komatsu *et al.*, 2003; Moreira *et al.*, 2003; Ramos *et al.*, 2010).

Following mutagenesis, mutants can be analysed for the alteration of a specific trait; often defined as a loss-of-function or gain-of-function phenotype. Mutants-of-interest may then be characterised and the genetic basis of the mutation determined. In many plasposon-based studies, the original protocol of rescue cloning was used to determine the site of the plasposon insertion within the genome. Briefly, this involves the digestions of genomic DNA of the plasposon mutant, self-ligation and transformation into a permissive *E. coli* host strain, followed by the isolation of the plasposon, and sequencing using outward facing primers targeting the plasposon (Dennis & Zylstra, 1998). Genomic flank-sequencing is a one-step alternative method to rescue cloning; suitable for scenarios where DNA fragments are not required for follow-up purposes (e.g. for sub-cloning or as a probe for Southern hybridisation). This method can obtain DNA adjacent to the site of insertion directly from the genomic DNA of plasposon mutants, using outward facing sequencing primers, that target the end of the plasposon, and arbitrary primers (Leveau *et al.*, 2006; Manoil, 2000). This method significantly reduces the time and effort involved in characterising a mutant library, and is therefore more amenable to high-throughput (Leveau *et al.*, 2006).

5.1.1 Aims

1) To investigate the genetic determinants of isothiazolone and DMDM hydantoin susceptibility of *B. lata* strain 383, by random insertion mutagenesis with the plasposon derivative pTn*Mod*O-Tp'.

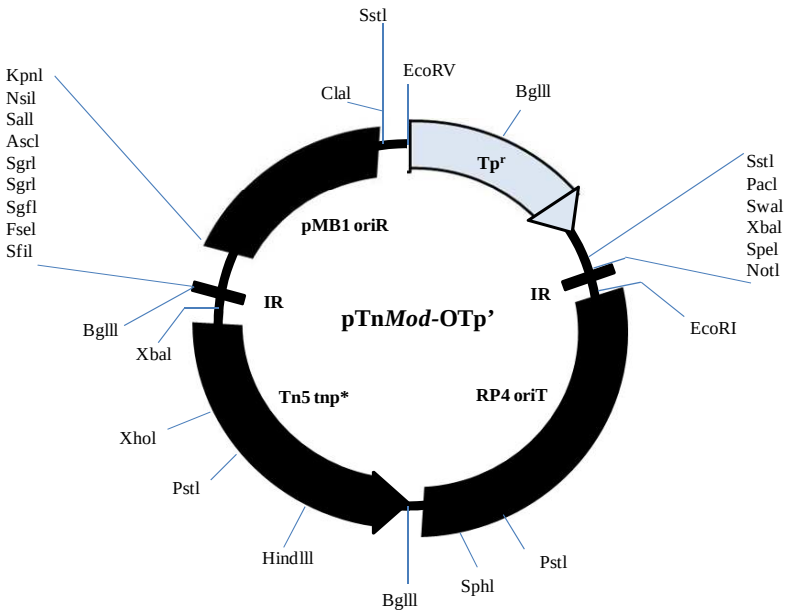


Figure 19.The plasposon pTnMod-OTp' used for the mutagenesis of *B. lata* strain 383.

The plasposon contains several modules (not to scale): a resistance gene – the schematic shows a trimethoprim resistance gene (Tp^r) as an open blue arrow; an origin of replication (pMB1 *ori*R), for replication in *E. coli* host such as DH5α or JM109; an origin of transfer, (RP4 *ori*T); a transposase gene outside the inverted repeats (Tn5 tnp*); and two multiple cloning sites. Commonly used restriction sites are labelled on the schematic. IR, inverted repeats. Adapted from Dennis *et al.* (1998) and Leveau *et al.*(2006).

5.2 RESULTS

Random transposon mutagenesis was used to investigate the genetic determinants of isothiazolone and DMDM hydantoin resistance of *B. lata* strain 383. A mutant bank of 2905 derivatives of *B. lata* strain 383 was created using the plasposon pTN*Mod*-OTp' (as described in section 2.10)To facilitate the rapid characterisation of mutants-of-interest, DNA sequences flanking the transposon insertion site (i.e. the transposon-chromosome junction) were produced directly from genomic DNA isolated from the plasposon mutants (as described in section 2.10.4). Two primers (P1 and P3) were designed for this purpose, to be used in conjunction with the published primers P2b and P4 (O'Sullivan *et al.*, 2007). To validate the specificity of primers P1 and P3, a third reverse primer (Pc) was designed; as the use of primer 2b in conjunction with primer P1 would have resulted in variably sized PCR product. The pairing of the designed primers P1 and P3 with Pc resulted in PCR amplicons of the expected size (458 bp and 426 bp, data not shown); therefore, the validated primers were used in conjunction with the published primers for all nested PCR reactions, and primer P3 was used for genomic flank-sequencing.

To test for randomness of transposon insertions, the bank was screened for auxotrophic mutants (as described in section 2.10.2). Auxotrophy can arise via the disruption of a number of genes encoding biosynthetic precursors, thereby providing a large target for mutagenesis (Hughes, 2007). Of the 2905 mutants, 38 (1.3%) were putative auxotrophs and these mutants were randomly distributed throughout the bank. In addition, ten of the 2905 mutants were selected at random, their identity confirmed by RAPD profile analysis, and the genetic basis of their mutation determined by correlation to the *B. lata* strain 383 genome (Winsor, 2008). The ten transposon insertions were located within different genes located on the first (n = 6), second (n = 2) and third (n= 2) chromosomes (data not shown).

The *B. lata* strain 383 transposon mutant bank was subsequently screened for altered susceptibility to DMDM hydantoin, methylisothiazolinone and the isothiazolone blend MIT/CMIT. Replica plating onto BSM (CYG) agar containing a range of preservative concentrations (as described in section 2.10.3) both above and below the *B. lata* strain 383 MIC was used to identify mutants with an increased or decreased susceptibility. This resulted in two classes of mutant being identified, which were designated as:

(i) those with a positive phenotype and an increased tolerance to a preservative;

(ii) those with a negative phenotype and an increased preservative susceptibility.

Mutants-of-interest, with a putative altered susceptibility, were rescreened for phenotypic

confirmation. The preservative screens identified a total of 67 (2.3%) mutants with altered susceptibility to that of the wild-type strain. The stability of the Tn mutants was proven by replating onto the preservative agents. The identity of these mutants was confirmed by RAPD profile analysis (data not shown), and the genetic basis of their mutation was determined. The 67 mutants identified were categorised based on the cluster of orthologous group (COG) assignment, as shown in Table 21.

For reasons of simplicity, henceforth the mutations will be described as the encoded protein homologue of the Tn disrupted genes; their actual roles in preservative resistance should still be considered putative at this stage.

Table 21.Transposon-interrupted genes of *B. lata* strain 383 exhibiting altered susceptibility to isothiazolone and/or DMDM hydantoin preservatives

Mutant categories (relating to COG assignment)	**Mutant name**	**Phenotype**	**Transposon-interrupted gene(s) ID**	**DNA flanking transposon insertion site (20 bp)**	**Putative gene product**
Amino acid transport and metabolism	16:H8	CMIT ^{T} MIT ^{T}	B2900	GATGTGCGTGGCCGAGCGGG	branched-chain amino acid efflux pump, AzlC (azaleucine resistance)
	5:D11	CMIT ^{S} MIT ^{S} DMH ^{S}	A5590	GAAGCCCGCCTTGATCAGCT	ketol-acid reductoisomerase
	27:H4	CMIT ^{S} MIT ^{S} DMH ^{S}	A5803	TGCCTGATGCCGCCCGACTA	sulfate adenylyltransferase subunit 2
Carbohydrate transport and metabolism	12:H10	DMH ^{T}	A5936 [a]	GCCGCGCGGCTGCCGGCCGA	Carbohydrate kinase, FGGY
	24:C3	DMH ^{T}	A5936 [a]	GCCGAAGGACTGGCTGCGCA	Carbohydrate kinase, FGGY
	12:C6	CMIT ^{S} MIT ^{S}	A3746	GGTGCGCTGTCGTGCGCGAA	phosphogluconate dehydratase
	26:C6	CMIT ^{S}	A6191 [b]	CGACCACGCCGTTCTCTTC	PTS system fructose subfamily IIA component
	24:C10	CMIT ^{S} MIT ^{S}	A5413 [c]	CGGCCTTCTCGATCGCCTGC	phosphopyruvate hydratase (enolase)
Cell division and chromosome partitioning	16:A3	CMIT ^{S}	A3275 [d]	ATCGAGGACNAGATCCACCG	tRNA uridine 5-carboxymethylaminomethyl modification enzyme, GidA
Cell envelope biogenesis, outer membrane	16:G9	DMH ^{S}	A5770	CGATCGCGACGCTCGCGTAA	surface antigen (D15)
	R:B9	CMIT ^{S}	A3741	TCGCGTGGACAGAACACGCT	Rare lipoprotein B
	8:E8	CMIT ^{S}	A3741	GGCACCGTGCTGATCCCGCC	Rare lipoprotein B
	29:G3	CMIT ^{S}	B1913	GGGCTGGGCTTCCAGCACGA	sugar nucleotidyltransferase-like
	31:G7	CMIT ^{S} DMH ^{S} MIT ^{S}	A3637	TCTCGACGCCGCGCGCCGCA	S-adenosyl-methyltransferase, MraW
	28:E10	CMIT ^{S}	A4275	GCCTCGTGCGCTGAGCACGG	mandelate racemase/muconate lactonizing protein
	12:E3	MIT ^{T}	A4646	AACTACATCGGCATCAAGAG	RND efflux system outer membrane lipoprotein, TolC
Intracellular trafficking and secretion	13:A9	MIT ^{T} DMH ^{T}	A3243	GCTCGACATCGCGGAGAAAC	General (type II) secretion system protein E, PulE
	31:F10	CMIT ^{S} DMH ^{S}	A3244	GCCGACCCGCGGACCAACTC	General type II and III secretion system protein D, PulD
	11:H2	MIT ^{T} DMH ^{T}	A3240	GGCACGATCAGCGCCGCCAG	General (type II) secretion pathway protein G, PulG
	12:A11	MIT ^{T} DMH ^{T}	A3240	TTTCACGCTGATCGAGATCA	General (type II) secretion pathway protein G, PulG
	15:A4	MIT ^{T}	A3235	TGCACATGGCCGGCCTTGTC	General (type II)secretion pathway protein L, PulL
	26:D12	CMIT ^{S}	A3536	GGTACAGCCCCGGCGCGACG	Sec-independent protein translocase, TatC
	14:G4	CMIT ^{S}	A3536	GTACCAGCACGAGAAGAAGC	Sec-independent protein translocase, TatC

Table 21. Continued

Mutant categories (relating to COG assignment)	**Mutant name**	**Phenotype**	**Transposon-interrupted gene(s) ID**	**DNA flanking transposon insertion site (20 bp)**	**Putative gene product**
Coenzyme metabolism	12:F11	CMIT ^{S}	A3375	GTTCGGCTTGAACGGGCCGT	S-adenosyl-L-homocysteine hydrolase
	30:C9	CMIT ^{S}	A6137	CATCTCGGCAGCGGCGTGCG	phosphopantetheine adenylyltransferase, coaD
	5:C4	CMIT ^{S} MIT ^{S} DMH ^{S}	A6230	GCCCGAGTGGATCCGCGTGA	lipoyl synthase
	21:G10	CMIT ^{S}	A6192	GGCCGAGGCGGCGCGGCGCG	glutathione synthetase
	17:H5	CMIT ^{S}	A6192	GCACGCGTACACCGCGTGCC	glutathione synthetase
Defense mechanisms	31:C2	CMIT ^{S}	A3192	GGGGTCAGGCGGCTTCCTCA	N-6 DNA methylase (Type II-modification system)
	24:A11	CMIT ^{S}	A3212	GCCGGCACACGTGCCGCACG	Type III restriction enzyme, res subunit
DNA replication, recombination, and repair	22:G4	CMIT ^{S}	B2218	CAGCGACGCGTCGCCCGAGA	DNA polymerase I, PolA
Energy production and conversion	16:E4	CMIT ^{S}	A3289	GGCGCGGCTCAGTGCCGGCT	F0F1 ATP synthase subunit epsilon
	21:F9	MIT ^{S}	A6182	ATCACGCACAGTGCGTCGTC	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
General function prediction only	27:F3	MIT ^{S}	A5887	GGTCAAGGACGTGCTGATCG	putative nucleotide-binding protein
	12:B2	CMIT ^{S}	A4597	GCTGGGCCGCCGAGACGTTC	predicted metallophosphoesterase
	30:F5	CMIT ^{S}	B0022	GCTCGAGCGTCACGACACCC	predicted RND efflux transporter
	9:H1	CMIT ^{S}	B1042	GATTCCGAATCGTGTCTTTC	Phage terminase, GpA
	30:A7	CMIT ^{S}	A5349	AATTGGTGATGAACATCACG	predicted lysophospholipid transporter , LplT (MFS H^{+} antiporter)
	26:H7	CMIT ^{S}	B2862	TCTCGCTGCTCGGCTTCGCC	Filamentous haemagglutinin-like
	27:H12	CMIT ^{S} MIT ^{S}	A4150	GCTGTTCGATCTGGACGGCA	predicted 2-phosphoglycolate phosphatase
Inorganic ion transport and metabolism	18:H7	CMIT ^{S}	A4832	ATCGACGTCGAATAGACGC	major facilitator transporter (Cyanate permease)
	23:G5	CMIT ^{S}	A5515	GAAGGCAATCGGCGTGAGAC	TonB-dependent siderophore receptor (Fe transport)
Lipid metabolism	29:B7	CMIT ^{S}	A5291	CGCCGACGGGACGTCGCCGC	6-phosphogluconate dehydrogenase, NAD-binding
	24:B11	CMIT ^{S}	A6047	GTCGACGACCGCGCGGGCGA	3-hydroxyacyl-CoA dehydrogenase
	24:D9	CMIT ^{S} MIT ^{S}	C6789	TCCAGGTACTGGATCAAATC	beta-hydroxyacid dehydrogenase

Table 21. Continued

Mutant categories (relating to COG assignment)	**Mutant name**	**Phenotype**	**Transposon-interrupted gene(s) ID**	**DNA flanking transposon insertion site (20 bp)**	**Putative gene product**
Nucleotide transport and metabolism / Amino acid transport and metabolism	27:A11	CMIT ^{S} MIT ^{S}	A6132	GCAACGCCGATCCTGCTGGG	ribose-phosphate pyrophosphokinase
Posttranslational modification, protein turnover, chaperones	4:H1	MIT ^{T}	B2297	TCGTCACGATCGGGACGGTA	OsmC-like protein (predicted redox protein)
	27:A12	MIT ^{S}	A4578	AGGTTTAAGAATTGTCAGAA	SirA-like protein (predicted redox protein)
	4:E4	CMIT ^{S} MIT ^{S}	A4439	GTTTACGGTGTTCAAGCAGTT	FtsH peptidase
	8:F12	CMIT ^{S} MIT ^{S}	A5331	CCGCGAAGGTGCTCGAGATG	PII uridylyl-transferase
Secondary metabolites biosynthesis, transport, and catabolism	18:A7	CMIT ^{S}	A3512	CGGTAGGAGTGGGGCTCACG	ABC transporter ATPase subunit
	11:E4	CMIT ^{S}	A3514	CTTCACGGCCGCACGCGGCT	ABC transporter, inner membrane subunit (Toluene tolerance)
Signal transduction mechanisms	12:F5	MIT ^{T}	B0028	GTAAAGGCGCCGCACGCCGT	diguanylate cyclase response regulator containing a cheY-like reciever and GGDEF domain
Transcription	26:G11	MIT ^{T}	A4647 [e]	CGCTTGCCCTCCCCTCGAAC	MarR family transcriptional regulator
	6:H7	MIT ^{T}	B2931	ATCCGGACGTCAGTGTCGA	LysR family transcriptional regulator
	3:E10	CMIT ^{S}	A5807 [f]	GGCGATCTCCGACTACTACG	LysR transcription factor, CysB
	2:F5	CMIT ^{S} MIT ^{S}	A6333	GCTCCAGATCGTCGAGAAGC	transcriptional regulator-like
	12:D4	CMIT ^{S} MIT ^{S}	C7481 [g]	GCGGCGCGTTGCCTCGAATT	transcriptional regulator-like
Translation, ribosomal structure and biogenesis	30:A4	CMIT ^{S}	A6478	GGCCGAGACCATGTATGCGG	peptide deformylase
	7:D11	CMIT ^{S}	unidentified	GTACAGATATTCCTCAGGGT	16S ribosomal RNA
	15:C9	CMIT ^{S} DMH ^{S}	unidentified	GGTTCTTAACTGAACCGAAA	16S ribosomal RNA
Unknown	4:A8	CMIT ^{T} MIT ^{T}	B2208	GCGCTTAAGCAGCCCGAGGA	hypothetical protein (extracellular localisation)
	21:A6	CMIT ^{S}	A3517 [h]	GACTTCGCGCTCGCGACGGT	hypothetical protein
	30:H9	CMIT ^{S} MIT ^{S}	A3517 [h]	CGTCCGCGCTCGCCGTGCTG	hypothetical protein
	24:H5	CMIT ^{S}	Intergenic [1]	GGTACGAGTGGGACCAGTGG	N/A
	24:E3	CMIT ^{S} MIT ^{S}	A5058	GTTCTAAGCTGCAGCTTGTG	hypothetical protein
	16:C5	CMIT ^{S}	Intergenic [2]	GAAGAAAATCGACTTCACTG	N/A

Footnotes:
Abbreviations: DMH, DMDM hydantoin; CMIT, isothiazolone blend of 3:1 methylisothiazolinone and chloromethylisothiazolinone; ^{S} increased susceptibility; ^{T} increased tolerance.
Flanking genes of interest: [a] A5935, a zinc-containing alcohol dehydrogenase ; [b] A6192 glutathione synthetase; [c] A5414 KdsA, 3-deoxy-D-manno-octulosonic acid (KDO) 8-phosphate synthase; [d] A3274 branched chain amino acid (ABC) transporter; [e] A4646_44 RND efflux , QacA family drug resistance; [f] A5808 branched chain amino acid transporter; [g] C7480 glutathione S-transferase -like protein; [h] A3516 ABC transporter involved in resistance to organic solvents . Brackets show multiple insertions within a gene. [1] Intergenic region upstream of gene B1475, a hypothetical protein with a predicted extracellular localisation; [2] Intergenic region upstream of gene B0512, a hypothetical protein.

In total, 62 genes were associated with preservative susceptibility; five of these mutants (24:C3, 8:E8, 17:H5, 14:G4, and 30:H9) had multiple transposon mutation insertions within the same gene (Table 21). All except one pair of mutants within the same gene demonstrated the same phenotype (Table 21). Paired mutants 21:A6 and 30:H9, with transposon insertion mutations of gene A3517 were the exception, with the former identified as a negative phenotype in the MIT/CMIT screen, while the latter was identified as a negative phenotype in both isothiazolone screens (Table 21).

5.2.1 Screening *B. lata* strain 383 mutant bank for altered susceptibility to DMDM hydantoin

The DMDM hydantoin screens identified a total of twelve mutants (0.41%) with an altered susceptibility to that of the wild-type strain (Table 21). Within these twelve mutants, five demonstrated a stable positive phenotype and seven displayed a stable negative phenotype; eight of the twelve mutants were also identified as having altered susceptibility in the isothiazolone preservative screens (Table 21). Mutants with altered susceptibility to DMDM hydantoin were associated with six COG categories, viz. amino acid biosynthesis (n=2); the outer membrane (n=2); secretion (n=4); translation (n=1); carbohydrate metabolism (n=2); and coenzyme metabolism (n=1).

The five mutants (12:H10, 24:C3, 11:H2, 12:A11 and 13:A9), identified as positive phenotypes in the DMDM hydantoin screens, had transposon insertion mutations within four genes located on the first chromosome of the *B. lata* strain 383 genome. Two mutants (12:H10 and 24:C3), identified only in the DMDM hydantoin screens, were interrupted in a carbohydrate kinase protein (gene A5936). This gene is adjacent to a zinc-containing alcohol dehydrogenase superfamiliy protein, with 95% homology to a glutathione-dependent formaldehyde dehydrogenase enzyme in *B. xenovorans.* Transposon insertion mutation of the gene A5936 may have caused polar effects altering the transcription of the neighbouring gene, which are more likely to have played a role in formaldehyde metabolism from its putative homology data (Hensel & Holden, 1996).

Three of the mutations (13:A9, 11:H2, and 12:A11) were mapped to two genes within an operon encoding core components of a type II protein secretion system *viz*. gene A3243, a cytoplasmic ATPase (PulE); and multiple insertions into gene A3240, encoding a major pseudopillin (PulG) (Table 21, Figure 20). An additional fourth mutation (31:F10), identified in the DMDM hydantoin screens, was mapped to a gene within this operon.

However, in contrast to the latter mutants, the disruption of the outer membrane secretin component (PulD) within the secretion system caused by 31:F10 resulted in an increase of susceptibility to DMDM hydantoin (Table 21, Figure 20).

In total, seven mutants (31:F10, 5:D11, 27:H4, 16:G9, 31:G7, 5:C4, and 15:C9) were identified in the DMDM hydantoin screens as having a negative phenotype. These had inserts in six genes distributed on the first chromosome of the *B. lata* strain 383 genome, with a seventh insert (15:C9) mapped to a 16SrRNA gene; the exact location of the 16S rRNA gene mutant could not be determined, being copies within the genome. Mutant 16:G9 was the only one of these mutants with a DMDM hydantoin negative phenotype; the other mutants were identified in both the DMDM hydantoin and isothiazolone screens The 16:G9 mutation mapped to a surface antigen protein with homology to the characterised protective surface antigen D-15 in *Haemophillus influenzae* (Thomas, 1995).

Four of the seven negative DMDM hydantoin phenotype mutants, demonstrated a greater than four-fold reduction in the MIC from wild-type levels. Mutation 5:D11, mapped to an oxidoreductases enzyme (Table 21) that participates in the biosynthesis of branched chain amino acids and coenzyme A; one of the main products of this enzyme is NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) a reducing agent involved in many processes including the regeneration of glutathione and the protection of important intracellular components against damage by reactive oxygen species. Mutation 27:H4 mapped to an enzyme that participates in sulphate assimilation and purine metabolism (Table 21) and plays an important role in protein synthesis. Mutant 5:C4 was interrupted in a lipoyl synthase encoding gene, an enzyme that participates in the biosynthesis of lipoic acid. In addition to its role as catalyst of metabolic reactions, lipoate has an important role in redox metabolism - effectively quenching harmful free-radicals and bridging cytoplasmic antioxidants, such as glutathione, with those that are membrane-bound (Spalding & Prigge, 2010). The fourth mutation to overlap DMDM hydantoin and isothiazolone screens, was mapped to MraW; an enzyme with S-adenosyl-methyltransferase activity which may participate in the biosynthesis of pepidoglycan (Carrión *et al.*, 1999).

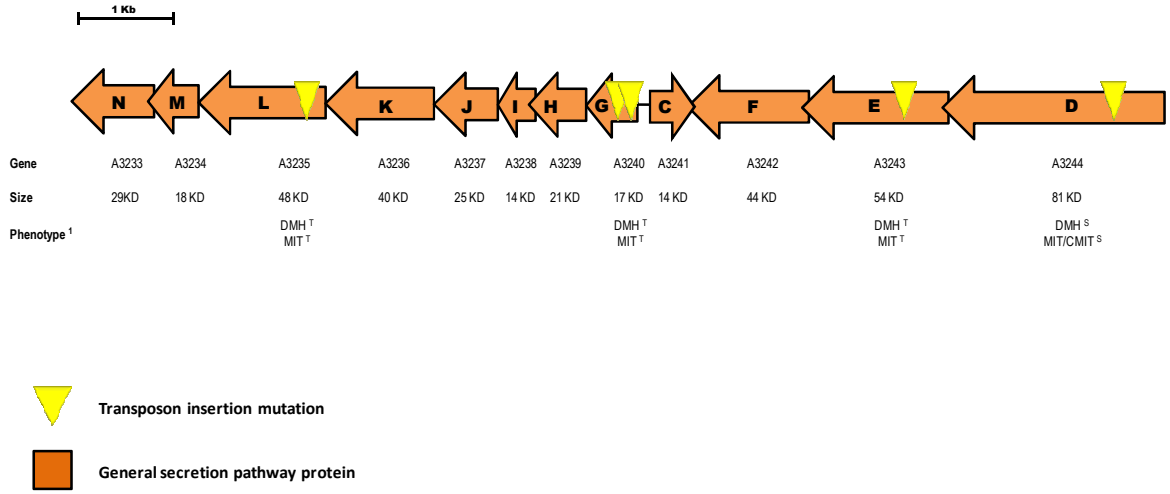


Figure 20. The transposon mutation with the general secretion pathway that altered the susceptibility of *B. lata* strain 383 to isothiazolone and DMDM hydantoin preservatives.

The schematic shows the arrangement of twelve genes (loci A3234_A3244), within an operon located on the 1st chromosome of the *B. lata* strain 383 genome, that encode proteins of a general type II secretion pathway; each drawn to the scale given by the bar on the left. The putative products of the genes are shown by the colour coded key, and the site of transposon-insertion mutation is labelled. [1] The mutant phenotype is indicated as: [T], increased preservative tolerance; and [S], increased preservative susceptibility. Transposon insertion mutation of genes encoding proteins E, G, and L (mutants: 13:A9, 12:A11, 11:H2, and 15:A4), resulted in a decrease in the susceptibility of *B. lata* strain 383 to methylisothiazolinone and DMDM hydantoin preservatives, as described in (Table 21). In contrast to the above, the interruption of the gene A3244 (mutant 31:F10), which encodes the putative protein D component of the secretion system, resulted in an increase in the susceptibility of *B. lata* strain 383 to the isothiazolone blend MIT/CMIT and DMDM hydantoin.

5.2.2 Screening *B. lata* strain 383 mutant bank for altered susceptibility to methylisothiazolinone

The methylisothiazolinone screens identified 29 mutants (0.99%) with altered susceptibility. Within these 29 mutants, 11 demonstrated a positive phenotype, whilst 18 demonstrated a negative phenotype (Table 21). Twenty of the 29 mutants with altered susceptibility to methylisothiazolinone were also identified in other preservative screens; seven overlapped with the DMDM hydantoin screen, and 14 overlapped with the isothiazolone MIT/CMIT screen. Mutants with altered susceptibility to methylisothiazolinone were associated with 12 COG categories, *viz.* secretion (n= 4); post-translation modification and chaperones (n=4); transcription (n=4); amino acid biosynthesis and transport (n= 3); unknown (n=3); the outer membrane (n= 2); carbohydrate metabolism (n= 2); general function prediction only (n=2); coenzyme metabolism (n=1); energy production (n= 1); lipid metabolism (n= 1); nucleotide transport and metabolism (n =1); and signal transduction mechanisms (n= 1). Mutants were interrupted in genes distributed throughout the three chromosomes of the of *B. lata* strain 383 genome: 23 on the first chromosome; five on the second chromosome; and two on the third chromosome.

Multiple mutations (13:A9, 11:H2, 12All, 15:A4) were mapped to protein components PulE, PulG and PulL of a type II general protein secretion pathway (Figure 20); all also had been previously identified in the DMDM hydantoin screens (section 5.2.1). The mutation of these genes within the operon resulted in the reduction of susceptibility of *B. lata* strain 383 to methylisothiazolinone and DMDM hydantoin.

Four mutations identified in the methylisothiazolinone screens were associated with posttranslational protein modification. Within this group, two mutations (4:H1 and 27:A12) were mapped to predicted redox proteins that participate in the regulation of disulfur bond formation (Winsor, 2008): the insert upstream of the OsmC-like protein resulted in a positive phenotype; whereas an insertion mutation of the SirA-like protein was associated with a negative phenotype. Mutation 4:E4 was mapped to a metalloendopeptidase , a proteolytic enzyme that plays a pivotal role in the quality control of membrane proteins under stress conditions (Ito & Akiyama, 2005 935). The fourth mutation, 8:F12, mapped to an enzyme that catalyses the uridylylation or deuridylylation of protein PII. This signal transduction protein plays a major role in the control of nitrogen

metabolism (Arcondéguy *et al.*, 2001). The interruption of this gene resulted in a greater than four-fold reduction in the MIC value from the wild-type level.

A second mutation associated with a signal transduction mechanism was also identified in the methylisothiazolinone screen. Mutation 12:F5 mapped to a diguanylate cyclase (PleD) response regulator containing a cheY-like receiver and GGDEF domain; disruption of this gene resulted in a decrease of MIT susceptibility. Diguanylate cyclases are responsible for the synthesis of cyclic-di-GMP, a ubiquitous global second messenger signalling molecule in bacteria, involved in a broad range of cellular processes (Paul *et al.*, 2007; Simm *et al.*, 2004).

Four mutations 26:G11, 6:H7, 2:F5 and 12:D4, were mapped to transcriptional regulators (Table 21). Mutations 26:G11 and 6:H7 had positive phenotypes, and both mapped to genes with putative roles in the regulation of transport systems. Mutant 26:G11 was interrupted in a MarR family transcriptional regulator gene A4647, a family of regulatory proteins associated with bacterial drug transporters (Grkovic *et al.*, 2002). Adjacent to the interrupted regulator was a RND efflux system with homology to EmrB/QacA-EmrA-TolC. Interestingly, mutation of the outer membrane lipoprotein (TolC) component of this efflux system (mutant 12:E3, gene A4646) resulted in a decrease of susceptibility to methylisothiazolinone. The second interrupted transcriptional regulator gene (mutant 6:H7, gene B2931) belonged to the LysR family of transcriptional regulator proteins, which had a close proximity to a multi-drug resistance RND efflux system homologous to EmrB/QacA-EmrA-TolC.

Three mutants (16:H8, 5:D11 and 27:H4), identified in the methylisothiazolinone screens, were associated with amino acid transport and metabolism (Table 21). 5:D11 and 27:H4 were oxidoreductase and sulphate metabolism mutants, also identified in the previous DMDM hydantoin screens. Mutant 16:H8, interrupted in a branched-chain amino acid efflux pump AzlC permease protein, was identified in isothiazolone screens as a negative phenotype. The hydrophobic AzlC permease protein has been shown to be involved in resistance to azaleucine, a toxic analogue of leucine (Belitsky *et al.*, 1997; Harrison *et al.*, 1975).

The remaining mutants were all identified as negative phenotypes in the methylisothiazolinone screens. Two of the mutations (12:C6 and 24:C10), hit genes involved in carbohydrate metabolism (Table 21), and were also identified in the

MIT/CMIT screen. The 12:C6 mutant mapped to an enzyme that participates in the Entner-Doudoroff pathway (glucose catabolism), a major source of reducing power and metabolic intermediates (Allenza & Lessie, 1982; Entner & Doudoroff, 1952). The 24:C10 mutation interrupted an enolase enzyme gene, involved in glycolysis. Two mutations were associated with the bacterial membrane. Mutant 21:F9 mapped to gene A6182, a glycerol-3-phosphate dehydrogenase (GpsA) that participates in glycerophospholipid metabolism; and a beta-hydroxyacid dehydrogenase mutant (24:D9), an enzyme with a functional role in lipid metabolism (Winsor, 2008). Mutations 5:C4 and 27:H12, were putatively associated with oxidative stress: the former was mapped to gene A6230 a lipoyl synthase (LipA), and the latter was mapped to a predicted phosphoglycolate phosphatise – a house cleaning enzyme that metabolises 2-phosphoglycolate produced in the repair of DNA lesions induced by oxidative stress (Pellicer *et al.*, 2003).

5.2.3 Screening *B. lata* strain 383 mutant bank for altered susceptibility to the blend of isothiazolone MIT/CMIT preservatives

The isothiazolone blend MIT/CMIT screens selected 52 mutants (1.79%) with a stable alteration of susceptibility, which was the largest number of mutants identified in a single preservative screen. Within this group, two mutants (16:H8 and 4:A8) demonstrated a positive phenotype, whilst 33 demonstrated a negative phenotype (Table 21). 19 of the 35 mutants, with altered susceptibility to MIT/CMIT, were also identified in the other preservative screens: six overlapped with the DMDM hydantoin screen and 17 overlapped with the methylisothiazolinone screen (Table 21).

Mutants with altered susceptibility to MIT/CMIT were associated with 18 COG categories, *viz*. general function prediction only (n= 6); unknown (n= 6); the outer membrane (n= 5); coenzyme metabolism (n= 5); secretion (n= 3); transcription (n= 3); amino acid biosynthesis and transport (n= 3); carbohydrate metabolism (n= 3); lipid metabolism (n= 3); translation, ribosome structure (n = 3); post-translation modification and chaperones (n= 2); secondary metabolites transport (n= 2); inorganic ion transport (n= 2); defence mechanisms (n= 2); energy production (n= 1); DNA replication, repair (n= 1); nucleotide transport and metabolism (n = 1); and cell division (n= 1) (Table 21). The mutants were interrupted in genes distributed throughout the *B. lata* strain 383 genome: 39 were located on the first chromosome; seven on the second chromosome; and two on the third

chromosome. Mutants 7:D11 and 15:C9 were interrupted in 16SrRNA genes whose exact location could not be determined (Table 21). Mutants 24:H5 and 16:C5, both with negative phenotypes, had insertion mutations in intergenic regions on the second chromosome of the *B. lata* strain 383 genome: the former mutation mapped to a region upstream of gene B1475, a hypothetical protein with 80% homology to a putative lipoprotein in *B. cenocepacia* J2315 (gene BCAM_1598); and the latter mutation identified upstream of B0512, a hypothetical predicted MFS permease protein (Winsor, 2008).

Four of the mutants (18:A7, 11:E4, 21:A6 and 30:H9), identified as negative phenotypes by the MIT/CMIT screens, were located in genes of an ATP-binding cassette (ABC) family transport system, with homology to the Ttg2ABC transporter system reported to be involved in resistance to organic solvents (García *et al.*, 2010; Winsor, 2008). These mutations were mapped to three components of the transporter: the ATPase subunit (Ttg2A); the periplasmic component (Ttg2C); and the hypothetical nucleotide triphosphate (NTP) binding protein with a STAS (sulphate transporter and anti-sigma factor antagonist) domain. Mutation of the inner membrane subunit gene A3514, and the hypothetical NTP binding protein gene A3517, resulted in a greater than four-fold reduction in the MIC value for methylisothiazolinone from the wild-type level.

Eight other mutations, identified in the MIT/CMIT screens, were associated with transport systems; this included the AzlC permease mutant that had been identified in the methylisothiazolinone screens (mutant 16:H8, Table 21). The remaining seven mutants were identified as negative phenotypes in only the MIT/CMIT screen (Table 21). Three of these mutations were located within components of inorganic ion transporters, *viz.* a cyanate permease protein (18:H7); an outer membrane TonB-dependent siderophore receptor protein, involved in the active uptake of iron (23:G5); and a predicted RND-type efflux transporter protein (30:F5). The mutation of the predicted RND-efflux protein gene B0022 resulted in a greater than four-fold reduction in the MIC value for MIT/CMIT than that of wild-type levels. A fourth mutation, 3:E10, was mapped to CysB, a LysR family transcriptional regulator reported to participate in the transportation and reduction of sulphate in Bcc bacteria (Iwanicka-Nowicka *et al.*, 2007). Three of the transport-related mutations (26:D12, 14:G4 and 31:F10) were associated with intracellular trafficking and secretion. Two of the three mutations assigned to this COG category, mapped to a single twin-arginine transport pathway protein TatC, an important component of a protein-targeting system dedicated to the transmembrane translocation of fully folded proteins

(Sargent, 2007). Mutant 31:F10 was interrupted in PulD, a secretion protein component of a general protein secretion pathway that had been identified in the previous methylisothiazolinone and DMDM hydantoin screens (Table 21, Figure 20).

Eight of the 52 mutants identified in the MIT/CMIT screen were associated with cell envelope biogenesis and the outer membrane, and lipid metabolism. Two mutations were mapped to genes involved in the biosynthesis or metabolism of membrane and cell envelope components, these included: a MraW mutant (31:G7), an enzyme involved in peptidoglycan biosynthesis; and a sugar nucleotidyltransferase-like mutant (29:G3), an enzyme involved in the production of polysaccharides. The peptidoglycan mutant (31:G7) was identified as putative general fitness mutant, after displaying a greater than four-fold reduction in the MIC for all three preservatives screens, whereas the nucleotidyltransferase mutant was unique to the MIT/CMIT screens. Two mutations were associated with the assembly and structure of the membrane bi-layer. Unique to the MIT/CMIT screen, two mutants (R:B9 and 8:E8) with a negative phenotype both disrupted an LptE encoding gene, a rare lipoprotein B formally known as RlpB. This lipoprotein has been shown to participate in the assembly of LPS in the leaflet of the outer membrane (Sperandeo *et al.*, 2008). A predicted LplT phospholipid flippase mutant (30:H7, gene A5349), with a negative phenotype, was also identified only in the MIT/CMIT screen. This enzyme facilitates the rapid transfer of phospholipids across the cytoplasmic membrane and is important in maintaining the structure and equilibrium of the membrane bi-layer (Pomorski & Menon, 2006).The interruption of this gene resulted in a greater than four-fold reduction in the MIC value for MIT/CMIT from wild-type levels. Mutations associated with lipid metabolism were also identified in the MIT/CMIT screen (Table 21); these included two beta-hydroxyacid dehydrogenase mutants (29:B7 and 24:D9), an enzyme involved in the D-galactarate metabolic process, and a hydroxyacyl–CoA dehydrogenase mutant (24:B11), an enzyme involved in multiple pathways including that of lipid metabolism(Winsor, 2008).

Five of the 52 mutations, identified in the MIT/CMIT screen, were associated with defence against oxidative stress and xenobiotics. Two mutations (21:G10 and 17:H5) were mapped to the same glutathione synthetase gene; an enzyme involved in the biosynthesis of glutathione and an essential component of the thiol redox system (Smirnova *et al.*). In addition, the mutation 26:C6 was mapped to a neighbouring gene upstream of the glutathione synthetase. All three mutants demonstrated an increase of susceptibility in the

MIT/CMIT screen. A second protein involved in redox was identified in the MIT/CMIT screen: mutant 5:C4 was disrupted in a lipoyl sythase, an enzyme involved in the biosynthesis of lipoate – a soluble molecule that quenches free-radicals. Interestingly, a homocysteine hydrolase mutant (gene A3375, 12:F11) was also identified in the screen as a negative phenotype by a greater than four-fold reduction in the MIC value for MIT/CMIT. Homocysteine hydrolase participates in the synthesis of cysteine (Kredich, 1996), which is a precursor of glutathione.

Four of the mutants, identified as negative phenotypes, were assigned to COG categories associated with the modification, replication, or repair of DNA. Two of these mutants (31:C2 and 24:A11) had disrupted defence mechanisms. One was a DNA methylase, which methylates DNA periodically throughout the genome in order to distinguish host DNA from foreign DNA (Cheng, 1995); the second was a type III restriction enzyme, that cuts single or double-stranded DNA at specific sites. Mutation 22:G4 was mapped to a DNA polymerase I enzyme, an important enzyme involved in DNA replication and repair (Madigan, 2003). PolA-deficiency can lead to the accumulation of single-strand gaps on the leading strand (O'Reilly & Kreuzer, 2004). Also associated with DNA repair, mutation 27:H12 was mapped to a predicted phosphoglycolate phosphatase, an enzyme involved in the metabolism of 2-phosphoglycolate produced in the repair of DNA lesions induced by oxidative stress (Pellicer *et al.*, 2003).

Four mutants, identified as negative phenotypes in the MIT/CMIT screen, were associated with COG categories relating to the translation or modification of proteins; two of these mutants (7:D11 and 15:C9) had inserts within 16S rRNA genes. The mutation 4:E4 was mapped to FtsH, a unique endoprotease that acts against intergral membrane-bound proteins, rapidly eliminating abnormal proteins (Ito & Akiyama, 2005). A peptide deformylase mutant (30:A4) was also identified; this metalloenzyme performs a critical cleavage step before further processing of newly synthesised protein can occur. The interruption of this gene resulted in a greater than four-fold reduction in the MIC value for MIT/CMIT from the wild-type level.

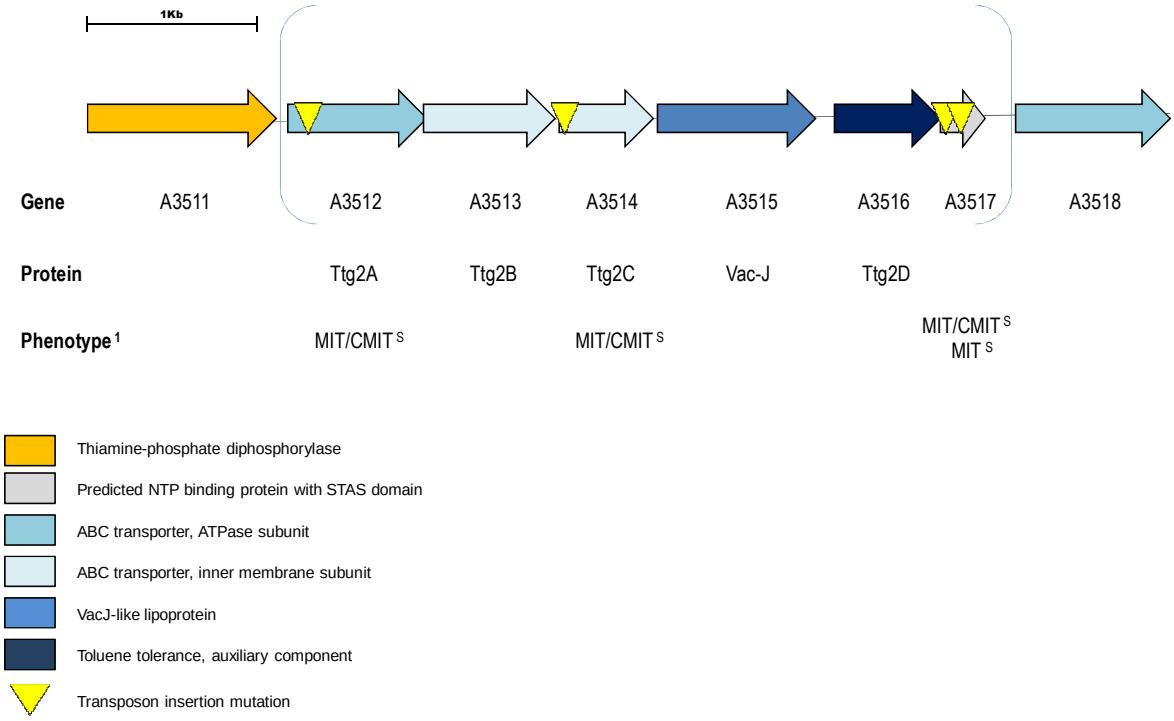


Figure 21. The transposon mutation of genes encoding an ABC-type transporter which altered the susceptibility of *B. lata* strain 383 to isothiazolone preservatives.

The schematic shows the arrangement of six genes within an operon, located on the 1st chromosome of the *B. lata* strain 383 genome, that encode proteins of an ABC-type transport system involved in resistance to organic solvents - each drawn to the scale given by the bar on the left. The putative products of the genes are shown by the colour coded key, and the site of transposon-insertion mutation is labelled. [1] The mutant phenotype is indicated as: [T], increased preservative tolerance; and [S], increased preservative susceptibility. The interruption of genes A3512, A3514 and A3517 (mutants 18:A7, 11:C4, 21:A6 and 30:H9) resulted in an increase in the susceptibility of *B. lata* strain 383 to the isothiazolone blend MIT/CMIT; in addition, mutation 30:H9, mapped to the hypothetical protein within the operon, resulted in an increase of susceptibility to methylisothiazolinone.

5.3 DISCUSSION

Transposon mutagenesis, using the plasposon Tn*Mod*OTp', successfully isolated *B. lata* strain 383 mutants with altered susceptibility to DMDM hydantoin, methylisothiazolinone and the blend of MIT/CMIT preservatives. Transposon mutants demonstrating an increase and decrease of preservative susceptibility were selected, and the genetic basis of the mutation was then determined by correlation to the *B. lata* strain 383 genome. The genetic characterisation of the mutants with altered preservative susceptibility identified multiple putative determinants of *B. lata* strain 383 preservative resistance, and did not suggest a single preservative target. This was perhaps reflective of the multi-factorial antimicrobial nature of the preservatives evaluated.

The roles of the identified determinants, in the resistance of *B. lata* strain to preservatives, are putative. They may be validated by complementing the Tn mutants with the wild-type gene, to restore the wild-type phenotype, or by creating site-directed/non-polar mutants.

5.3.1 The genetic determinants associated with DMDM hydantoin resistance

Formaldehyde, and formaldehyde donors such as DMDM hydantoin, are extensively used in a variety of settings for disinfection, antisepsis, and preservation purposes (Russell, 2004a). Bacterial resistance to these agents is well documented (Chapman, 1998), and has been associated with formaldehyde dehydrogenase enzymes, as well as changes to the outer membrane of some Gram-negative bacteria (Kaulfers & Brandt, 1987; Wollmann & Kaulfers, 1991). Four different pathways for the detoxification of formaldehyde are known in bacteria, three of which have been characterised in *Burkholderia* species: these include the NAD-linked glutathione (GSH)-independent formaldehyde dehydrogenase, and NAD-linked GSH-dependent formaldehyde oxidation systems (Gutheil *et al.*, 1997; Marx *et al.*, 2004).

The mutagenesis of *B. lata* strain 383 identified twelve genes putatively involved in susceptibility to the formaldehyde releasing agent DMDM hydantoin. Within this group five mutants were identified with a decreased level of susceptibility to that of the wild-type. Two of these mutants were interrupted in gene A5936, a carbohydrate kinase adjacent to a zinc-containing alcohol dehydrogenase enzyme, with high homology to a GSH-dependent formaldehyde dehydrogenase in *B. xenovorans* (Chain *et al.*, 2006). An increase in the synthesis of formaldehyde dehydrogenases has been associated with an increased resistance to formaldehyde agents in *P. aeruginosa* species (Orth *et al.* 2006).

The substrate for GSH-dependent formaldehyde dehydrogenase is hydroxymethyl glutathione: the adduct formed between formaldehyde and the SH group of the intracellular nucelophillic tripeptide, glutathione (Gutheil *et al.*, 1997; Hopkinson *et al.*, 2010). It is noteworthy that the glutathione synthase mutants (21:G10 and 17:H5), identified in the isothiazolone screens, were not identified in the DMDM hydantoin screen. To test the putative role of the zinc-containing alcohol dehydrogenase enzyme (gene A5935) as a DMDM hydantoin resistance determinant, a directed mutagenesis approach could be applied using a recently published system for the creation of targeted, non-polar unmarked gene deletions in *Burkholderia* (Flannagan *et al.*, 2008). However, studies suggest that *Burkholderia* may possess multiple formaldehyde detoxification pathways that may contribute to formaldehyde tolerance (Marx *et al.*, 2004 741). The mutation, and consequential disruption, of a single pathway involved in formaldehyde detoxification may therefore be compensated by other systems.

A type II (T2S) protein secretion system (orthologous to BCAL3515_3527 (Holden *et al.*, 2009)) was also identified as a putative DMDM hydantoin resistance determinant by the mutagenesis screen. Protein secretion by this system is a two-step process, whereby proteins are first translocated across the inner membrane by the Sec or Tat pathway, and then transported from the periplasm to the extracellular environment via a secretin protein localised in the outer membrane (Cianciotto, 2005). Type II secretion systems are required for the secretion of a variety of enzymes (e.g. proteases, lipases, chitinases, cellulases and nucleases), toxins, and virulence factors (Cianciotto, 2005; Fehlner-Gardiner *et al.*, 2002; Salmond & Reeves, 1993; Somvanshi *et al.*, 2010). In *B. lata* strain 383, transposon mutation of a T2S cytoplasmic ATPase (PulE) component, and the major pseudopilin (PulG), that facilitates the attachment of the ATPase to the inner membrane, resulted in a decrease in susceptibility to DMDM hydantoin and methylisothiazolinone preservatives.

The presence of eleven genes, for core secretory proteins (T2S CDEFGHIJKLMN) within the putative *B. lata* strain 383 operon, suggests that the secretion system is functional (Cianciotto, 2005). As mutation of the core protein components of the inner membrane platform reduced the preservative susceptibility of *B. lata* strain 383, it would suggest that a defective secretory pathway is associated with increased preservative tolerance. As the core membrane platform components (i.e. the ATPase, pseudopillins and protein L) are known to be essential for T2S function (Somvanshi *et al.*, 2010). Interestingly, an orthologous T2S system operon in *B. cenocepacia* J2315 was observed to be down-

regulated during growth in sputum (Drevinek *et al.*, 2008). Sputum has multiple antimicrobial compounds which may act in a similar way to the preservatives studied. Whereas the interruption of the T2S inner membrane platform reduced susceptibility, interruption of the outer membrane secretin protein gene increased susceptibility. The secretin protein is responsible for the final translocation of proteins out of the periplasm to the extracellular milieu. The observed increase of susceptibility may have been a result of a detrimental accumulation of exoproteins in the periplasm (Wandersman, 1992) or lack of an essential secreted preservative resistance factor.

Until recent years the study of T2S systems has focused on the secretion of virulence factors and their role in pathogenicity (Cianciotto, 2005). Along with the expansion of research into the role of T2S in environmental niches, their role as putative determinants of Bcc bacteria preservative resistance is worthy of further investigation.

5.3.2 The genetic determinants associated with methylisothiazolinone resistance

In addition to the identification of the previously discussed T2S system, transport proteins were identified as putative determinants of methylisothiazolinone resistance. The insertion mutation of two transcriptional regulators of the MarR and LysR family, in close proximity to RND type efflux systems (EmrB/QacA-EmrA-TolC), resulted in a reduction of the susceptibility of *B. lata* strain 383 to methylisothiazolinone. Transcriptional regulators of the MarR and LysR families are known to control expression of genes encoding bacterial drug efflux components (Grkovic *et al.*, 2002), by functioning as a repressor or activator of transcription. Mutation of the repressor or activator has been associated with the overproduction of efflux components and enhanced efflux of antimicrobials. For example, the production of an inactive MexR repressor protein (of the MarR family) in *P. aeruginosa*, has been described to result in the over-expression of the MexAB-OprM pump complex, with the enhanced efflux of a range of antibiotics including fluoroquinolones, β-lactams and tetracycline (Srikumar *et al.*, 2000).

The interruption of the coding region of the LysR family regulator gene B2931 in *B. lata* strain 383, suggests the regulator may function as a putative repressor of the neighbouring RND system. Interestingly, a mutation upstream of the MarR family transcriptional regulator gene A4647 and the interruption of the 53.28 kDa outer membrane protein TolC gene (A4647) of the adjacent RND system, decreased susceptibility to methylisothiazolinone. The mutation of the last gene within the putative operon would be

unlikely to have resulted in the over-production of efflux components, thereby enhancing efflux. This would suggest that the decreased expression of this specific pump, or perhaps an alteration in the outer membrane protein profile or uptake of the preservative agent, was associated with decreased susceptibility. Similarly, the interruption of a 26.25 kDa outer membrane permease protein (AzlC), of a branched amino acid transporter, resulted in a decrease of susceptibility to methylisothiazolinone.

The mutation of a 57.8 kDa diguanylate cyclase (PleD), an enzyme involved in the synthesis of the second messenger signalling molecule ci-di-GMP in bacteria (Jenal & Malone, 2006), resulted in a decrease of the susceptibility of *B. lata* strain 383 to methylisothiazolinone. PleD is a member of the response regulator family of two-component signal transduction systems, that contains a cheY-like receiver domain and a highly conserved GGDEF domain (also referred to as a DGC or DUF1 domain)(Chan *et al.*, 2004). The association of the GGDEF domain with a sensory input domain couples internal or external stimuli to a specific cellular response (Paul *et al.*, 2007). Upon phosphorylation of the receiver domain, the activated PleD catalyses the conversion of two molecules of GTP to ci-di-GMP (Chan *et al.*, 2004). The transposon mutagenesis of *B. lata* suggests that a determinant, or determinants, of methylisothiazolinone resistance may be regulated by ci-di-GMP. Although many aspects of ci-di-GMP signalling remain uncharacterised, it has been shown to regulate an array of cellular processes associated with antimicrobial resistance such as the transition from sessility to motility; the production of exopolysaccharides; surface attachment of cells; motility; and aggregation (Jenal & Malone, 2006; Simm *et al.*, 2004).

In addition to electrophillic attack, research suggests the intracellular generation of radicals may be an antimicrobial mechanism of isothiazolone agents (Chapman *et al.*, 1993). Bacteria possess multiple mechanisms to protect themselves from toxic reactive species, generated endogenously through metabolism or through exposure to oxidising agents. Sir-A-like and an OsmC-like predicted redox proteins, putative regulators of disulfide bond formation, were identified as putative determinants for methylisothiazolinone resistance. The interruption of the coding region of a SirA-like predicted redox protein, and an insertion upstream of the OsmC-like predicted redox protein, resulted respectively in an increase and decrease of *B. lata* strain 383 preservative susceptibility. The identification of a predicted 2-phosphoglycolate phosphatase mutant in the methylisothiazolinone screens, suggests that methylisothiazolinone may also induce DNA lesions via oxidative stress.

Phosphoglycolate phosphatase mutants are shown to accumulate 2-phosphogylcolate, which forms intracellularly through DNA repair machinery (Pellicer *et al.*, 2003).

5.3.3 The genetic determinants associated with isothiazolone blend (MIT/CMIT) resistance

An ATP-binding cassette (ABC) family transporter system, with homology to the Ttg2ABC transporter involved in multidrug resistance and toluene tolerance in *Pseudomonas* species (García *et al.*, 2010), was identified as a key determinant of *B. lata* strain 383 resistance to the isothiazolone blend MIT/CMIT. Four mutants, with disrupted Ttg2 transport proteins, demonstrated an increased susceptibility to the isothiazolone blend. The ABC-transporter operon comprised of six genes encoding: an ATPase subunit (Ttg2A); an ABC-transporter inner membrane permease subunit (Ttg2B); an ABC-transporter inner membrane subunit (Ttg2C); a VacJ-like lipoprotein; an ABC-transporter auxiliary component (Ttg2D); and a hypothetical protein, predicted to be an NTP binding protein with a STAS domain (sulphate transporter and anti-sigma antagonist(Aravind & Koonin, 2000)). Mutants interrupted in the inner membrane subunit (Ttg2C) and the latter predicted NTP binding protein, displayed a greater than four-fold increase in the MIC value for MIT/CMIT in preservative screens. This suggests the ABC-transporter may be involved in decreasing intracellular accumulation of the isothiazolone preservatives. NTP binding proteins with a STAS domain in association with anion transporters, have been shown to localise to the cytoplasmic portion of the transporter (Aravind & Koonin, 2000). Therefore, findings suggest that the putative efflux of MIT/CMIT may be regulated by intracellular concentrations of a signalling molecule or stimuli.

The mutation of a second transporter protein gene (B0022), belonging to the RND family, was associated with a large increase in the susceptibility of *B. lata* to the isothiazolone blend MIT/CMIT. The interrupted 92.4 kDa transport protein had a 61% homology to the organic solvents ABC transporter permease Ttg in *Cupriavidus necator*. Adjacent to the interrupted coding region were other efflux complex components, *viz.* a vacJ-like lipoprotein and a Ttg2D auxiliary toluene tolerance protein. Prior to the discovery of the ABC-family toluene tolerance transporter Ttg2ABC in *P. putida* (García *et al.*, 2010), the majority of efflux pumps for organic solvents identified in Gram-negative bacteria belonged to the RND family (Ramos *et al.*, 2002). This suggests that putative organic solvents efflux systems from the ABC and RND family may be involved in the efflux of MIT/CMIT in *B. lata* strain 383.

Electrophillic agents, such as isothiazolones, non-specifically attack cellular nuceolphiles such as protein sulfhydryls (Chapman *et al.*, 1993). Glutathione is an intracellular tripeptide that protects protein sulfhydryls by presenting a non-competing non-lethal target. Glutathione-deficient bacteria may display hypersusceptiblity to electrophilic agents (Chapman *et al.*, 1993). The mutagenesis of *B. lata* strain 383 revealed glutathione to be a putative determinant of susceptibility to the isothiazolone blend MIT/CMIT: as the interruption of the coding region of a glutathione synthase, and the mutation of a gene flanking a glutathione transferase, resulted in increased isothiazolone susceptibility. Putatively in connection with glutathione, the interruption of a homocysteine hydrolase, an enzyme that participates in the synthesis of the glutathione precursor cysteine, also resulted in an increase of susceptibility.

Oxidative-stress defence mechanisms (OsmC and SirA) were identified as putative determinants of *B. lata* strain 383 resistance to methylisothiazolinone but not for the blend of isothiazolones MIT/CMIT. To counter oxidative stress, cells express proteins that detoxify the cell and repair the induced damage (Storz & Imlay, 1999). Interestingly, although predicted redox protein mutants were not identified, a large number of mutations putatively associated with the repair of induced damage were identified in the MIT/CMIT screens. These mutations were associated with the synthesis and assembly of membrane components, protein synthesis, DNA repair or the metabolisms of bi-products of DNA repair machinery.

5.4 CONCLUSIONS

The main conclusions from this chapter are as follows:

1. *B. lata* strain 383 resistance to the formaldehyde releasing agent DMDM hydantoin may involve its detoxification by glutathione-dependent formaldehyde dehydrogenase;

2. a type II general secretory pathway was identified as a putative *B. lata* strain 383 determinant of susceptibility to DMDM hydantoin and methylisothiazolinone preservatives. Defective secretion by this system caused by Tn insertion was associated with a decrease in the preservative susceptibility of the *B. lata* strain;

3. two multi-drug resistance RND type efflux pumps, with homology to EmrB/QacA-EmrA-TolC complex systems, were putatively identified as determinants of *B. lata* strain 383 susceptibility to methylisothiazolinone. The interruption of LysR and MarR family transcriptional regulators, in close proximity to the coding region of the efflux systems, also resulted in a decrease of susceptibility;

4. the mutation of PleD, a protein with a GGDEF diguanylate cyclase domain involved in the synthesis of ci-di-GMP molecules, suggested that a determinant or determinants of *B. lata* strain 383 resistance to methylisothiazolinone may be regulated by the second messenger signalling molecule;

5. two transporter systems, belonging to the ATP-binding (ABC) and RND families, with homology to transporter systems involved resistance to organic solvents (Ttg2) in *Pseudomonas* species, were identified as putative determinants of *B. lata* strain 383 susceptibility to the blend of isothiazolone preservatives (MIT/CMIT);

6. glutathione was identified as a putative determinant of *B. lata* strain 383 susceptibility to the blend of isothiazolone preservatives (MIT/CMIT), protecting protein sulfhydryls by presenting a non-lethal target to the electrohillic preservative agents;

7. proteins associated with countering the affects of oxidative stress (e.g. the regulation disulfide bond formation, lipid metabolism and DNA repair), were identified as putative determinants of *B. lata* strain 383 susceptibility to isothiazolone preservatives.

6 GENE EXPRESSION OF *B. LATA* STRAIN 383 IN RESPONSE TO PRESERVATIVES

6.1 INTRODUCTION

The regulation of gene expression occurs primarily at the transcriptional level and is one of the main mechanisms involved in initiating adaptive processes in a cell. Coupled with translation, changes in transcription can lead to the production of new proteins and changes in the cellular machinery (Van Vliet, 2010; Wildsmith & Elcock, 2001). The detection and quantification of RNA transcripts is a powerful means of gaining knowledge of cellular functions and processes, and over the years various methods of transcript profiling have been developed. In recent decades, the traditional step-by-step study of the regulation and function of individual genes and proteins has been superseded by technologies that enable the simultaneous analysis of multiple genes. DNA microarrays (DNA chips), developed in the 1990s during the genomic revolution, have become a popular method for large scale gene expression measurement (Wildsmith & Elcock, 2001). The expression level of thousands of genes, expressed by an organism under certain conditions, can be determined by the extraction and reverse transcription of mRNA into cDNA, followed by labelling and hybridising the cDNA to probes complementary to every gene within the organism, that are attached to a solid support usually made of glass (Harrington *et al.*, 2000). This technology is capable of extracting vast amounts of information about the transcriptome, which represents the complete collection of transcribed sequences of a cell that can be used to gain insight into gene function, expand our knowledge of cellular processes, identify potential drug targets, conduct genomic comparisons (including the evolutionary classification of bacterial strains), and to capture a genome-wide snap-shot of transcriptional activity in response to a specific stimulus (Harrington *et al.*, 2000; Leiske *et al.*, 2006).

Within the last ten years, there has been a substantial growth in the number of *Burkholderia* species genomes sequenced and made publicly available (Mahenthiralingam, 2007). One of the many benefits of this excellent dataset is that it has enabled the development of whole-genome microarrays for the study of Bcc species. As a result of its clinical significance, *B. cenocepacia* strain J2315 (Vandamme *et al.*, 2003), a virulent epidemic CF pathogen from the ET12 lineage, was the first Bcc pathogen to be selected for genome sequencing (Holden *et al.*, 2009), and following its public release the complete J2315 genome became the basis for the first custom microarray of the *B. cenocepacia* species (Leiske *et al.*, 2006). The *B. cenocepacia* microarray has since been successfully used to profile global gene expression changes in response to several environmental stimuli, and for comparative genomics. For example, Drevinek *et al.* (2008) used a genomic array approach to characterise the global gene expression changes in response to growth in CF sputum. This revealed that genes associated with antimicrobial resistance, iron uptake, protection against oxygen, secretion and motility were among the most altered in sputum. Recently, Sass *et al.* (2011) used the same trancriptomic strategy to examine antimicrobial resistance traits of *B. cenocepacia* J2315 by profiling global gene expression changes in response to antibiotics, and spontaneous *B. cenocepacia* mutants with elevated antibiotic resistance. The global mapping of genetic pathways, which mediate antibiotic resistance in the *B. cenocepacia* strain, revealed that they are multi-factorial in nature. Interestingly, spontaneous resistance involved more gene expression changes than that observed in response to sub-MIC antibiotic exposure; and the altered gene expression in the resistant mutants was stable irrespective of the presence of the priming antibiotic (Sass *et al.*, 2011). The microarray data identified known and novel efflux genes, antibiotic degradation/modification systems, and membrane function, as determinants of resistance. The transcriptomic analysis identified potential therapeutic targets and a means potentially to improve the efficacy of current antibiotics used to treat CF infection.

The Agilent 60-mer SurePrint technology, which has been extensively validated on *B. cenocepacia* (Drevinek *et al.*, 2008; Leiske *et al.*, 2006), has since been used in the design of custom microarrays for other Bcc species. Recently, Mahenthiralingam *et al.* (2011) successfully employed a custom microarray, designed to the *B. ambifaria* AMMD genome, to map genes that contribute to the biosynthesis of enacyloxins on a global scale. In the present study, a transcriptomic microarray-based approach was used to investigate genes and gene pathways involved in Bcc preservative resistance, and those associated with preservative-induced adaptive resistance, using a custom gene expression microarray designed to the *B. lata* strain 383 genome. The *B. lata* strain 383 genome-sequenced reference strain (www.JGI.doe.gov) represented a Bcc species commonly encountered in the environmental-industrial niche (Chapter 3, Section 3.2.1), and an isothiazolone-adapted derivative with elevate preservative resistance, had also been isolated after its progressive sub-culture in the presence of sub-inhibitory preservative concentrations (Chapter 4). Profiling global gene expression changes in *B. lata* strain 383 provoked via exposed to sub-inhibitory preservative concentrations, and after preservative-induced adaptive resistance was used to provide a novel insight into the molecular mechanisms involved in Bcc preservative resistance. The overall goal was to identify putative resistance targets, and to facilitate the implementation of preservative strategies to better target these organisms in industry.

6.1.1 Aims

The aims of this chapter were to employ a transcriptomic, microarray-based strategy to investigate the following:

1. How exposure to sub-inhibitory concentrations of DMDM hydantoin and the blend of isothiazolone preservatives (MIT/CMIT), affects gene expression in *B. lata* strain 383.

2. How preservative-induced adaptive resistance to the blend of isothiazolone preservatives (MIT/CMIT) alters gene expression of *B. lata* strain 383.

3. How exposure to sub-inhibitory concentrations of the isothiazolone blend (MIT/CMIT) affects gene expression of the preservative-adapted *B. lata* strain 383-CMIT derivative.

6.2 RESULTS

6.2.1 Growth curve analysis and RNA extraction

To ensure consistent RNA profiling, cells were harvested during the mid-logarithmic growth phase. It was therefore necessary to ascertain suitable sub-inhibitory preservative concentrations that would allow logarithmic growth. Growth curve analysis revealed that 0.00162% DMDM hydantoin and 0.00001498% MIT/CMIT were suitable sub-inhibitory concentrations for microarray analysis. When cultured in the presence of preservatives at these levels, *B. lata* strain 383 and its preservative-adapted derivative 383-CMIT reached a mid-logarithmic growth phase, and the target optical density for harvesting (0.5 at 600nm), within seven hours (in contrast to between five and six hours for preservative-free medium). *B. lata* strain 383 cultured in medium containing preservative concentrations above these levels (i.e. closer to half that of the MIC of *B. lata* strain 383) failed to reach the target optical density for harvest within 9 hours.

6.2.2 Microarray performance and data analysis

Five microarray experiments were conducted:

(1) *B. lata* strain 383 in a preservative-free medium;
(2) *B. lata* strain 383 in BSM (CYG) medium containing 0.00162% DMDM hydantoin;
(3) *B. lata* strain 383 in BSM (CYG) medium containing 0.00001498% MIT/CMIT;
(4) the preservative-adapted *B. lata* strain 383-CMIT derivative in a preservative-free medium;
(5) *B. lata* strain 383-CMIT derivative in a BSM (CYG) medium containing 0.00001498% MIT/CMIT.

Each of the five experimental conditions were performed as three biological replicates; therefore, 15 arrays were analysed in total. Microarray data were analysed using the statistical software Genespring GX version 7.3 (as described in section 2.9.12). Cluster analysis of the replicate microarray data demonstrated that the transcriptomic dataset was suitable for further analysis. The similarity of gene expression profiles between the five experimental conditions is shown as a condition tree in Figure 22. The *B. lata* strain 383

microarrays clustered together, as did the two arrays for the preservative-adapted *B. lata* strain 383-CMIT derivative.

After the removal of internal control genes, and the normalisation of data, analysis was performed using a list of 7749 coding sequences of the *B. lata* strain 383 genome. A 1.5-fold change filter criterion was applied to array comparisons using a confidence filter of $P<0.05$. This generated four lists of genes:

(i) genes with differential expression in response to DMDM hydantoin (conditions 1 and 2);

(ii) genes with differential expression in response to MIT/CMIT (conditions 1 and 3);

(iii) genes with differential expression in the preservative-adapted *B. lata* strain 383-CMIT derivative (conditions 1 and 4);

(iv) *B. lata* strain 383-CMIT genes with differential expression response to the MIT/CMIT (conditions 4 and 5).

Gene lists are shown in the following sections. For reasons of simplicity, henceforth, the differentially expressed genes will be described as their encoded protein homologue. However, their functions and roles in preservative resistance should still be considered putative at this stage.

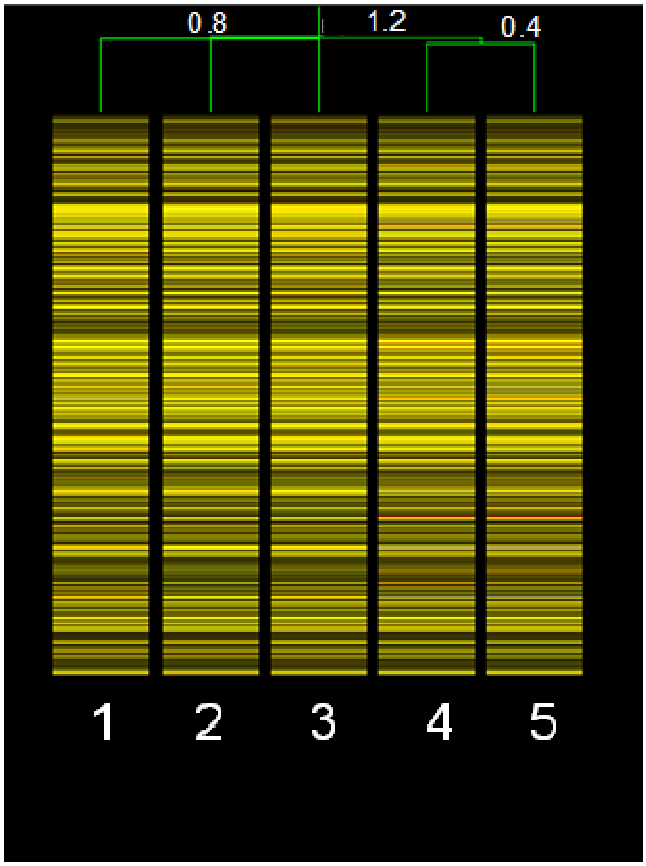


Figure 22. Condition tree analysis of the five microarray experimental conditions.

The condition tree analysis of microarray experimental conditions: (1) *B. lata* strain 383 wild-type, cultured in preservative-free BSM (CYG) medium; (2) *B. lata* strain 383 wild-type, in BSM (CYG) medium containing 0.00162% DMDM hydantoin; (3) *B. lata* strain 383 wild-type, in BSM (CYG) medium containing 0.00001498% of the isothiazolone blend MIT/CMIT; (4) *B. lata* strain 383-CMIT (a preservative-adapted derivative), cultured in preservative-free BSM (CYG) medium; (5) *B. lata* strain 383-CMIT, cultured in BSM (CYG) medium containing 0.00001498% of the isothiazolone blend MIT/CMIT. Each experimental condition is an average of triplicate replicate microarray experiments. In the condition tree, the array conditions (1-5) are grouped according to the degree of similarity of their expression profiles over the selected probes: where a correlation distance of 1 represents identical microarrays. The array-to-array correlation analyses were as follows: conditions 1, 2 and 3 = 0.8; conditions 4 and 5 = 0.4; conditions 1, 2 and 3 with 4 and 5 = 1.2.

6.2.3 The validation of observed *B. lata* strain 383 microarray data

In order to validate the microarray results, four genes (B1004, B1327, A6485 and A3516) with significantly higher levels of expression in the preservative-adapted *B. lata* strain 383-CMIT, were examined using quantitative real-time PCR (qRT-PCR) (Table 22). The expression of two genes (B0668 and A3949) observed to be down-regulated in the microarray was examined by semi-quantitative PCR (Figure 23). Quantitative PCR data was normalised using the expression levels of a housekeeping gene (*recA*), a control gene without altered expression under these conditions. Expression of the gene *phaC*, another control gene without altered expression, was used as a control for semi-quantitative PCR.

The selected genes represented a range of expression changes observed in the preservative-adapted *B. lata* strain 383 microarray (-6.3 to +25 fold). Quantitative PCR demonstrated that the expression levels of the four genes, observed to have significantly increased 1.9 to 25-fold in the microarray experiment, in the preservative-adapted derivative, were consistently up-regulated from that of the parental strain (Table 22). Expression analysis of these genes correlated well with the results observed by microarray analysis: gene B1004, with a 25 fold change by microarray analysis had a 482 fold change by qRT-PCR; A3156, with a 1.9 fold change by microarray analysis had a 1.74 fold change by qRT-PCR.

The differential expression of the selected genes B0668 and A3949, observed to have been down-regulated (6.3 and 4.5 fold) by microarray analysis, could not be quantified by qRT-PCR due to limited sensitivity. Semi-quantitative PCR was therefore used to examine the differential expression of these genes, a method that was successfully employed in the validation of previous microarray expression data (Drevinek *et al.*, 2008). Using pooled cDNA from the microarray experiments, gene B0668 was consistently amplified from *B. lata* strain 383 (microarray condition 1) within 30 cycles, along with the control gene *phaC* (Figure 23). In contrast, gene B0668 was not consistently amplified within 30 cycles for the preservative-adapted derivative (microarray condition 4) (Figure 23); this corroborated the microarray data, showing that gene B0668 was down-regulated in the adapted strain. While the control gene *phaC* was consistently amplified within 30 cycles, amplified product for gene A3949 could not be visualised for either strain, suggesting very low levels of expression in both the parental and adapted strain.

Table 22.Validation of the *B. lata* strain 383-CMIT gene expression by quantitative real-time PCR

		Microarray		qRT-PCR quantification	
Gene Name	**Putative gene function**	**Fold change**	**P-value**	**$2^{-\Delta\Delta}$ CT**	**Pfaffl**
B1004	Secretion protein, RND-type efflux system	25	0.00038	832.20 (±143.40)	482.59 (±78.93)
B1327	MFS transporter protein	4.6	0.000749	178.94*	105.47*
A6485	Phenylacetic acid degradation-related protein	2.8	0.000303	7.06 (±0.54)	7.70 (±0.27)
A3516	ABC-type transporter, auxillary component	1.9	0.00151	1.93 (±0.11)	1.74 (±0.03)

Footnotes: Quantitative rt-PCR values represent the mean fold change (±STDEV) of two biological replicates (six technical replicates in total) in comparison with the transcription level in the parental *B. lata* strain 383. * fold change calculated from one biological replicate (three technical replicates). Data are normalised to the expression levels of the *recA* reference gene A5979.

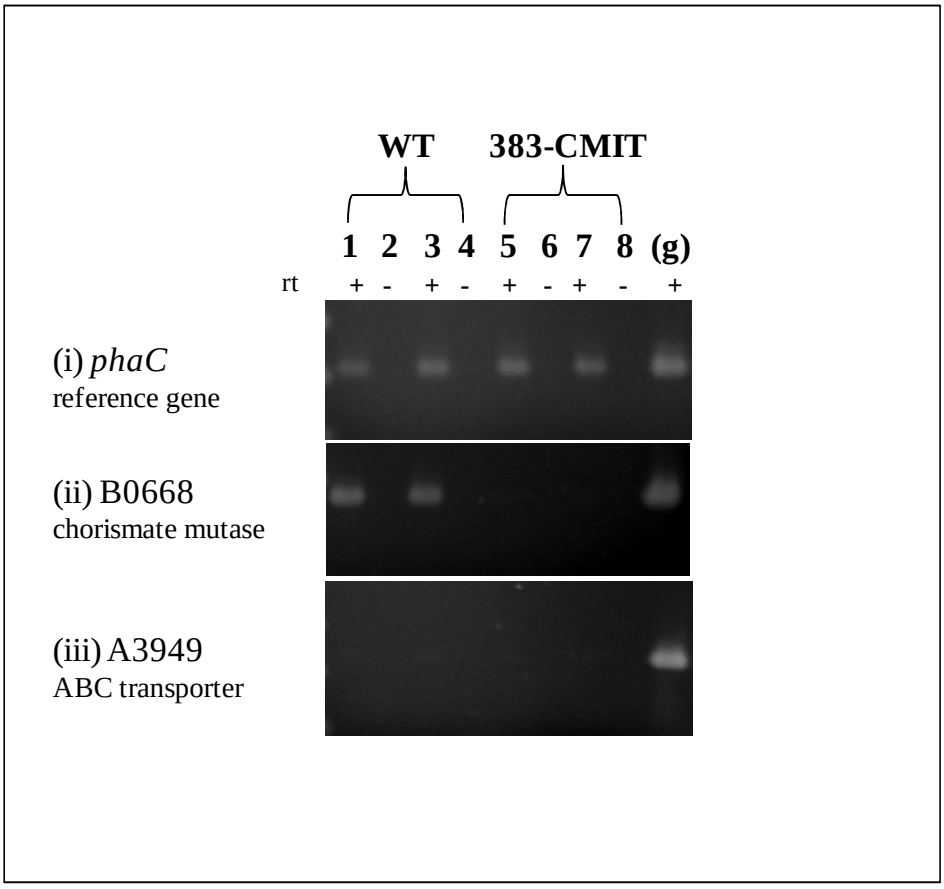


Figure 23. The validation of microarray observed *B. lata* strain 383-CMIT gene expression by semi-quantitative PCR.

The results of semi-quantitative RT-PCR on cDNA derived from two biological replicates of *B. lata* strain 383 (lanes 1- 4), and its preservative-adapted derivative *B. lata* strain 383-CMIT (lanes 5-8), grown on preservative-free medium; with genomic DNA (g) as a positive control. The PCR products, amplified after 30 PCR cycles, are shown for the following genes: (i) *phaC* (A5090; a control gene without altered expression); (ii), a chorismate mutase (B0668; down-regulated 6.3 fold in the preservative-adapted derivative) ; and (iii) the inner membrane sub-unit of an ABC-type transporter (A3949; down-regulated 4.5 fold in the preservative-adapted derivative).

6.2.4 Differential *B. lata* strain 383 gene expression in response to DMDM hydantoin

Microarray analysis of *B. lata* strain 383 cultured sub-inhibitory concentrations of DMDM hydantoin preservative, demonstrated that 305 coding genes were differentially expressed with a 1.5 fold filter applied; this represented 3.9% of all CDSs annotated in the *B. lata* strain 383 genome. Filtering with a confidence level of $P<0.05$, the list was reduced to 47 genes: 15 up-regulated (Table 23) and 32 down-regulated ≥1.5-fold (Table 24). Within this list, four genes were significantly up-regulated ≥2-fold and ten were significantly down-regulated ≥2-fold. The observed maximum fold-change in expression was 5.6-fold.

The differentially expressed genes were disseminated throughout the *B. lata* strain 383 genome with 14, 27 and 5 genes located respectively on the first, second and third chromosomes. The distribution of the 47 genes with significant differential expression among COG categories is shown in Figure 24. Genes were associated with 13 COG categories involved in metabolism (n= 18), cellular processes and signalling (n= 13), information storage and processing (n= 3) or were poorly characterised in function (n= 12).

B. lata strain 383 genes, observed to have been up-regulated ≥2-fold, in response to DMDM hydantoin exposure, were involved in metabolism or outer membrane biosynthesis. These included a zinc-containing alcohol dehydrogenase, 5.6- fold; a short chain dehydrogenase, 3-fold; hydroxphenylacetate hydroxylase, 2-fold; and a glucose phosphosphate uridyltransferase, 2.6-fold (Table 23). Three outer membrane porin genes located on the second chromosome, that encoded proteins putatively 38 to 40 kDa in size, were up-regulated >1.6-fold. Genes within a putative operon (B1863_B1869), encoding an ABC-type transporter system were also up-regulated (>1.6-fold) in response to exposure to the formaldehyde releasing agent (Table 23).

A larger number of *B. lata* strain 383 genes (n= 32) were significantly down-regulated in response to sub-inhibitory concentrations of DMDM hydantoin. Ten genes were observed to have been down-regulated ≥2-fold; these genes were associated with amino acid transport, signal transduction, lipid metabolism, inorganic ion transport, the outer membrane, or were poorly characterised (Table 24). Within this group, amino acid transporter genes C7548 and B196, were down-regulated respectively by a 3.7 and 2.6-fold. An outer membrane porin (gene B0225), putatively 40 kDa in size, was down-

regulated 2.1-fold. Two genes encoding signal transducers were also down-regulated greater than 1.5-fold. These included a LuxR family regulator with a cheY-like receiver (gene B0664, down-regulated 2.1-fold) and a diguanylate cyclase gene (C7110) with GGDEF/EAL domains (down-regulated 2.0-fold).

A third putative signal transducer gene, encoding a cheA homologue, was also observed to be down-regulated (1.6-fold) in response to DMDM hydantoin (Table 24). CheA is a signal transduction histidine kinase central to the signal transduction pathway for chemotaxis (Hess *et al.*, 1988). Also associated with motility, a flagellar hook-associated protein FlgK gene (A6361) with homology to BCAL0576 in *B. cenocepacia* J2315, was down-regulated 1.7-fold. Other flagellar structural or associated genes were found to have altered in expression by up to 1.3-fold or remained unaffected; however, these genes were excluded from the final gene list because of the filter criteria applied across the data set.

Table 23. Up-regulated gene expression in *B. lata* strain 383 exposed to DMDM hydantoin

Gene Name	Fold change	p-value	Putative gene function
A6455	5.6	0.00291	Zinc-containing alcohol dehydrogenase superfamily
B1023	1.8	0.00272	outer membrane protein (porin)
B1861	1.8	0.00476	transcriptional regulator, AraC family
B1863 [a]	1.6	0.022	Peptidase M55, D-aminopeptidase
B1867 [a]	1.8	0.0409	ABC dipeptide/oligopeptide/nickel family transporter, periplasmic ligand binding protein
B1869 [a]	1.7	0.00389	Peptidase T2, asparaginase 2
B1873	1.7	0.0471	outer membrane protein (porin)
B2109	2.6	0.0375	UTP-glucose-1-phosphate uridylyltransferase
B2233	1.7	0.037	hypothetical protein
B2334	1.5	0.000602	hypothetical protein
B2410	2.0	0.0309	4-hydroxyphenylacetate 3-hydroxylase
B2594	1.6	0.0297	transcriptional regulator, LysR family
B2596	1.6	0.00873	outer membrane protein (porin)
C6836	1.8	0.0127	Phytanoyl-CoA dioxygenase
C7587	3.0	0.0151	short chain dehydrogenase

Footnotes:

All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a > two-fold change in expression are shown in bold

[a] genes within a predicted operon

Table 24. Down-regulated gene expression in *B. lata strain* 383 exposed to DMDM hydantoin

Gene Name	Fold change	p-value	Putative gene function
A3772	-1.6	0.0125	hypothetical protein
A4309	-2.5	0.0189	phosphatase-like
A4382	-1.6	0.0403	hypothetical protein
A4541	-2.4	0.0494	hypothetical protein
A4545	-1.9	0.021	Phosphoesterase
A5091	-1.5	0.045	acetyl-CoA acetyltransferase
A5481	-1.5	0.00573	transcriptional regulator, histidine utilization repressor, GntR family
A5767	-1.8	0.0245	OmpA/MotB family protein
A6101	-2.0	0.0469	Acetoacetyl-CoA reductase
A6102	-1.5	0.0327	acetyl-CoA acetyltransferase
A6103	-1.6	0.0157	Phasin
A6244	-1.6	0.0162	5'-Nucleotidase-like
A6361	-1.7	0.0237	flagellar hook-associated protein, FlgK
B0225	-2.7	0.0228	outer membrane protein (porin)
B0424	-1.9	0.0241	Propionyl-CoA carboxylase
B0664	-2.1	0.0267	transcriptional regulator, LuxR family
B0683	-1.5	0.049	Beta-lactamase
B1177	-1.6	0.0423	Short-chain dehydrogenase/reductase SDR
B1561	-2.4	0.0359	hypothetical protein
B1561	-1.6	0.00471	hypothetical protein
B1750	-1.6	0.0237	Glycerophosphoryl diester phosphodiesterase
B1877	-1.6	0.0269	Metallophosphoesterase
B1961	-2.6	0.0188	glutamine ABC transporter periplasmic-binding protein
B1977 [a]	-1.6	0.0435	hypothetical protein
B1979 [a]	-1.6	0.0242	putative CheA signal transduction histidine kinase
B1981	-1.6	0.0476	protein of unknown function DUF485
B2478	-1.5	0.0198	outer membrane protein (porin)
B2852 [b]	-2.1	0.029	Alkaline phosphatase
B2853 [b]	-1.7	0.00296	Alkaline phosphatase
C6744	-1.8	0.0356	Acyl-CoA dehydrogenase
C7110	-2.0	0.0408	diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains)
C7548	-3.7	0.0216	Amino acid transporter

Footnotes:

All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a > two-fold change in expression are shown in bold

[a] genes within a predicted operon

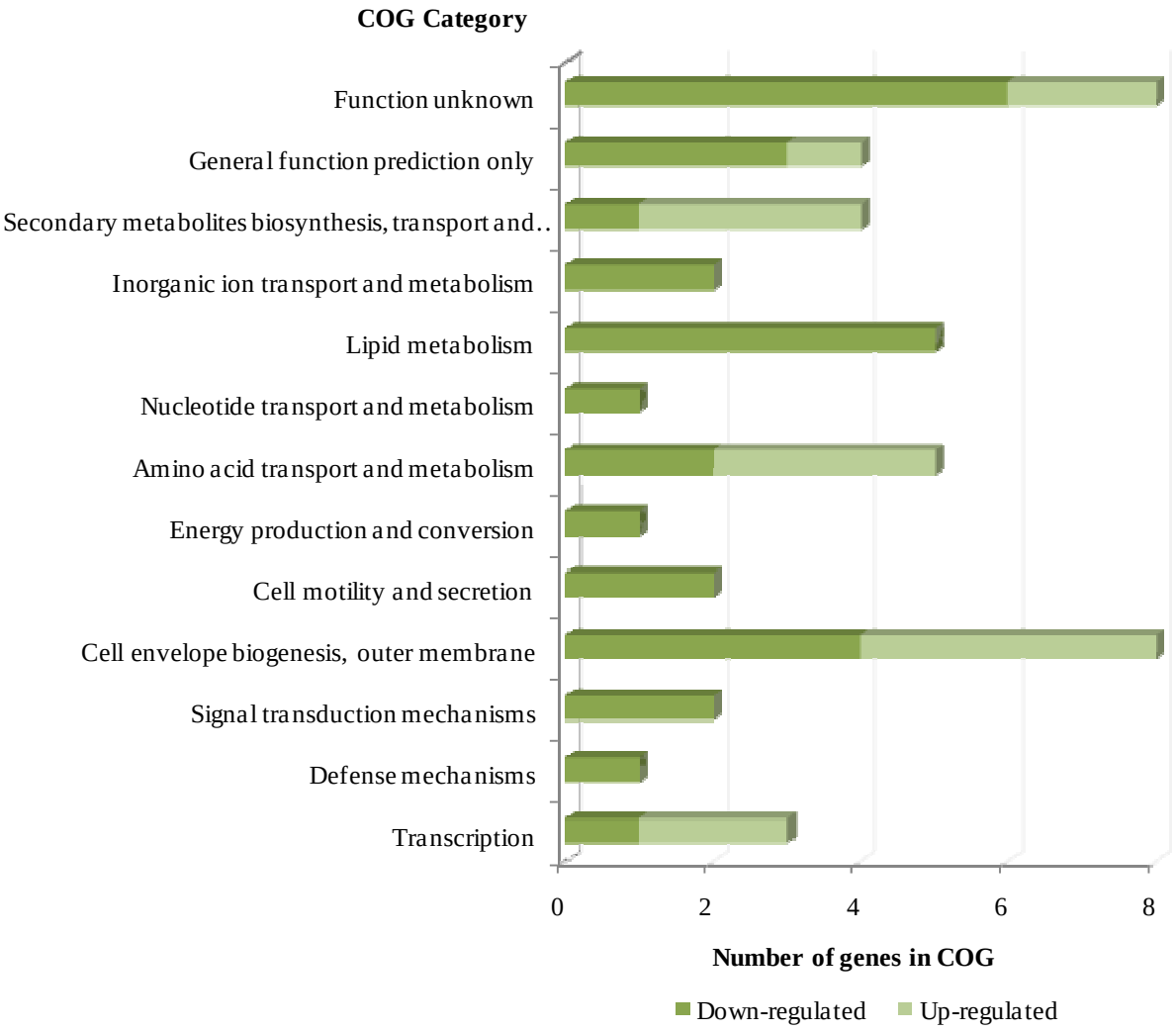


Figure 24. The distribution of *B. lata* strain 383 genes, differentially expressed in **response to DMDM hydantoin exposure, among COG categories.**

The dark green and light green bars correspond to 47 genes whose expression significantly decreased or increased (respectively) by at least 1.5 fold ($P < 0.05$), in response to exposure to sub-inhibitory concentrations of the preservative DMDM hydantoin.

6.2.5 Differential *B. lata* strain 383 gene expression in response to the isothiazolone preservative blend MIT/CMIT

Microarray analysis, of *B. lata* strain 383 cultured in sub-inhibitory concentrations of the cosmetics-grade blend of isothiazolone preservatives MIT/CMIT, demonstrated that 298 coding genes were differentially expressed with a 1.5-fold filter applied; this represented 3.8% of all CDSs annotated in the *B. lata* strain 383 genome. Filtering with a confidence level of $P < 0.05$, the list was reduced to 27 genes, with 21 up-regulated (Table 25) and 6 down-regulated ≥ 1.5-fold (Table 26). Within this list, six genes were significantly up-regulated ≥ 2-fold and three were significantly down-regulated ≥ 2-fold. The observed maximum change in expression was 4.4-fold.

The differentially expressed genes were disseminated throughout the *B. lata* strain 383 genome, with 6, 12 and 9 genes located respectively on the first, second and third chromosome. The distribution of the 27 genes with significant differential expression among COG categories is shown in Figure 25. Genes were associated with 11 COG categories involved in metabolism (n= 10), cellular processes and signalling (n= 3), information storage and processing (n=2), or were poorly characterised in function (n=12).

B. lata strain 383 genes observed to have been up-regulated ≥2-fold in response to MIT/CMIT exposure, were putatively involved in outer membrane biosynthesis, metabolism, or were poorly characterised. These included a capsule polysaccharide biosynthesis gene (C7396, 2.6-fold), a dihdropyrimidime dehydrogenase gene (C6656, 2.6-fold), a peptidase S10 gene (B0371, 2.3-fold), and an integral membrane protein that putatively participates in regulating cation conductance (gene B2416, 2-fold) (Table 25). Genes up-regulated greater than 1.5-fold putatively encoded a zinc-containing alcohol dehydrogenase (gene A6455) that had been observed to be up-regulated in response to DMDM hydantoin, and a phase terminase GpA protein (gene B1042;1.6-fold), that had been identified previously as a putative determinant of *B. lata* strain 383 MIT/CMIT resistance in the transposon mutagenesis screens (Chapter 5).

Only three *B. lata* strain 383 genes were observed to have been down-regulated greater than or equal to 2-fold, in response to MIT/CMIT exposure. These putatively encoded a short-chain alcohol dehydrogenase (gene C7132; 2-fold), involved in metabolism, and two genes putatively involved in the metabolism and transport of amino acids (C7548 and B1364; 3.3-, 4.4-fold) (Table 26).

Table 25. Up-regulated gene expression in *B. lata* strain 383 exposed to MIT/CMIT isothiazolone blend

Gene Name	Fold change	p-value	Putative gene function
A3419	1.7	0.041	hypothetical protein
A4956	1.6	0.0492	putative aldehyde dehydrogenase
A5329	1.5	0.0235	30S ribosomal protein S2
A5720	1.6	0.0165	hypothetical protein
A6316	1.5	0.00227	transcriptional regulator, LysR family
A6455	1.8	0.0169	Zinc-containing alcohol dehydrogenase superfamily
B0371	2.3	0.0319	Peptidase S10, serine carboxypeptidase
B1025	1.8	0.0155	hypothetical protein
B1042 $^{Tn\Delta}$	1.6	0.0231	Phage terminase GpA
B1301	2.0	0.0268	hypothetical protein
B1872	1.9	0.0137	Alpha-glucosidase
B1873	1.6	0.0488	outer membrane protein (porin)
B2416 [a]	2.0	0.0173	SPFH domain-containing protein/band 7 family protein
B2417 [a]	2.1	0.0418	hypothetical protein
B2823	1.9	0.0485	hypothetical protein
C6645	1.9	0.00407	Aldehyde dehydrogenase
C6656	2.6	0.028	dihydropyrimidine dehydrogenase
C7082	1.7	0.0373	Acyl-CoA dehydrogenase-like
C7208	1.7	0.0458	hypothetical protein
C7370	1.7	0.0203	hypothetical protein
C7396	2.6	0.0443	Capsule polysaccharide biosynthesis

Footnotes:

All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a > two-fold change in expression are shown in bold

$^{Tn\Delta}$ Transposon mutant (9:H2) , interrupted in gene B1042, demonstrated an increase of susceptibility in the MIT/CMIT screens, as discussed in chapter 5.

[a] genes within a predicted operon

Table 26. Down-regulated gene expression in *B. lata* strain 383 exposed to MIT/CMIT isothiazolone blend

Gene Name	Fold change	p-value	Putative gene function
B1771	-1.5	0.0201	Zinc-containing alcohol dehydrogenase superfamily
C6744	-1.7	0.000664	Acyl-CoA dehydrogenase
B1177	-1.7	0.0335	Short-chain dehydrogenase/reductase SDR
C7132	-2.0	0.0107	Short-chain dehydrogenase/reductase SDR
C7548	-3.3	0.0233	Amino acid transporter
B1364	-4.4	0.00111	D-serine dehydratase

Footnotes:

All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a > two-fold change in expression are shown in bold

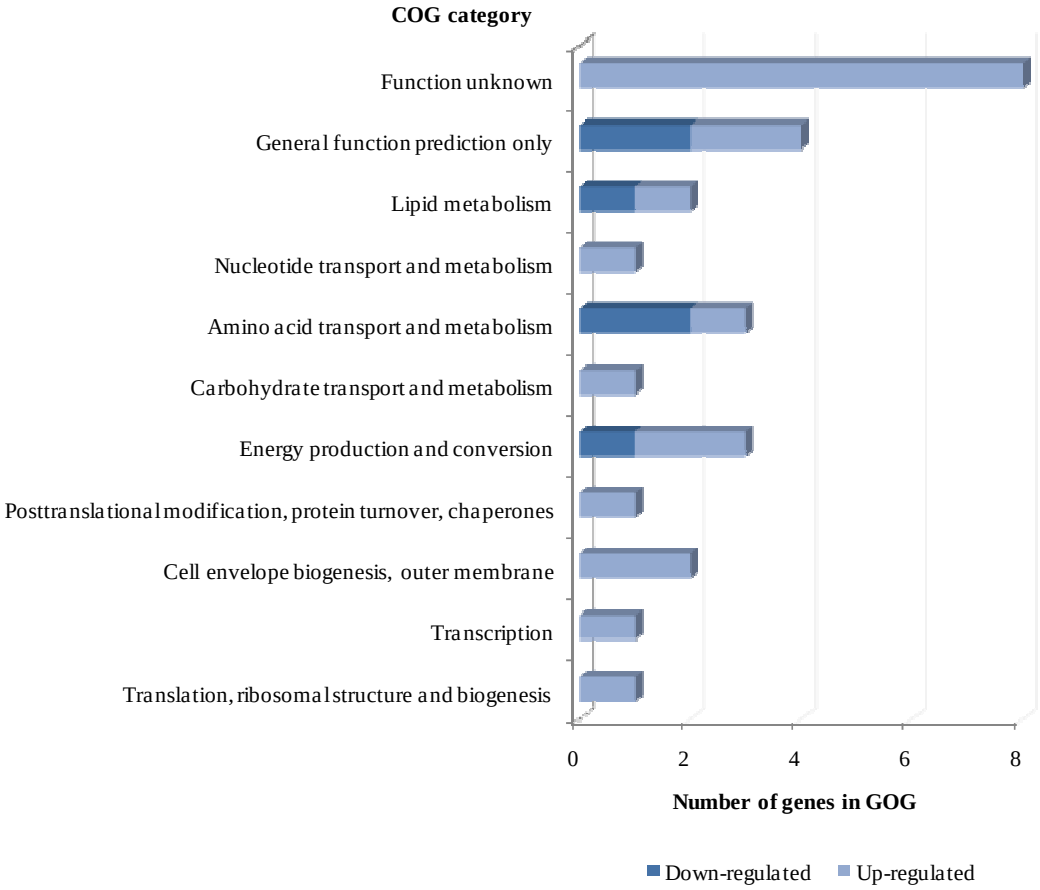


Figure 25. The distribution of *B. lata* strain 383 genes, differentially expressed in response to MIT/CMIT isothiazolone exposure, among COG categories.

The dark green and light blue bars correspond to 27 genes whose expression significantly decreased or increased (respectively) by at least 1.5 fold ($P < 0.05$), in response to exposure to sub-inhibitory concentrations of the isothiazolone preservative blend MIT/CMIT.

6.2.6 Differential gene expression in the preservative-adapted *B. lata* strain 383-CMIT derivative

Microarray analysis, of the preservative-adapted *B. lata* strain 383-CMIT derivative, cultured in a preservative-free medium, demonstrated that 529 coding genes were differentially expressed with the 1.5-fold filter applied; this represented 6.8% of all CDSs annotated in the *B. lata* strain 383 genome. Filtering with a confidence level of $P<0.05$, the list was reduced to 216 genes: 126 significantly up-regulated (Table 27) and 90 significantly down-regulated ≥1.5-fold (Table 28). Within this list, 37 genes were significantly up-regulated ≥2-fold, and 29 were significantly down-regulated ≥2-fold. The expression of seven genes had significantly altered more than 5-fold in the preservative-adapted *B. lata* strain 383 derivative.

The differentially expressed genes were disseminated throughout the *B. lata* strain 383 genome, with 81, 103 and 32 genes located respectively on the first, second and third chromosome. The distribution of the 216 genes with significant differential expression among COG categories is shown in Figure 26. Genes were associated with 19 COG categories involved in metabolism (n= 80), cellular processes and signalling (n= 31), information storage and processing (n= 16) or were poorly characterised (n= 89).

The largest fold-change in the expression of *B. lata* strain 383-CMIT genes, was observed for a tripartite RND efflux system encoded on the second chromosome (Figure 27): expression of the HlyD family secretion protein component increased by 25-fold, the outer membrane lipoprotein increased 10-fold, while expression of the hydrophobe/amphiphile efflux protein increased 9.3-fold (Table 27).

In total, the expression of twelve putative transporters had increased >1.5-fold in the preservative-adapted strain (Table 27), four of these belonging to the ABC-family. The expression of five genes within the putative operon A3512_A3517 increased between 1.7- to 2.1-fold. This operon encodes a putative ABC-type transport system homologous to the Ttg2 system that is associated with tolerance to organic solvents in *P. aeruginosa*. This ABC-type transport system was identified as a putative determinant of *B. lata* isothiazolone resistance in the mutagenesis study (Chapter 5). Expression of an ABC Fe^{3+} siderophore transporter protein gene (B1768) had increased 4.5-fold, whilst expression of two other ABC transporter systems had increased 2.1 to 2.3-fold (genes C7504, B0540 and

B0541) in the adapted derivative (Table 27). The expression of three major facilitator superfamily transporter proteins (involved in carbohydrate transport) and two amino acid transporter protein genes had also altered between 1.8 to 4.7-fold (genes A4968, B1327, B0355, A5849 and C7469, Table 27). Expression of a homologue of an EmrB/QacA family drug-resistance transporter protein gene (A5867) was also up-regulated 1.6-fold in the preservative-adapted derivative. The expression of three putative outer membrane porin proteins had increase by 1.6 to 1.9-fold (Table 27) in the preservative-adapted derivative. These putative porin proteins ranged from 38 to 40 kDa in size.

Three stress response protein genes were observed to be significantly up-regulated ≥1.9-fold in the preservative-adapted *B. lata* strain 383-CMIT (Table 27). Within this group of genes, B0269 and B0270 (up-regulated 2.1 and 1.9-fold) were putative signal transduction mechanisms within a putative operon involved in resistance to tellurite (Taylor, 1999; Winsor, 2008). A periplasmic superoxide dismutase protein SodB gene -A5874, orthologous to BCAL2757 in *B. cenocepacia* J2315 - was observed to be up-regulated (2.6-fold) in the isothiazolone-adapted strain (Table 27). Only a minor significant change in the expression (1.2-fold) was observed for the copper/zinc binding superoxide dismutase SodC homologue (gene A5765) (see www.ebi.ac.uk/arrayexpress E-MEX-2827). In contrast, a homologue of a peroxidase (gene A3905) was observed to have been significantly down-regulated (2.8-fold) in the adapted derivative (Table 28).

Several of the genes observed to be down-regulated were putatively associated with the shikimate pathway that participates in the synthesise of the three proteinogenic aromatic acids and a broad range of mostly aromatic secondary metabolites including some siderophores(Dosselaere & Vanderleyden, 2001). The largest fold change (-6.3-fold) was observed for a homologue of a chorismate mutase gene (B0688). This had 73% homology to the salicylate biosynthesis protein PchB in *P.aeuriginosa* PA01 (Winsor, 2008) involved in the synthesis of the siderophore pyochelin (Gaille *et al.*, 2002). A second gene within the putative operon, encoding an AMP-synthetase and ligase, was also significantly down-regulated by 4.3-fold (Table 28) in the preservative-adapted derivative. A third gene B0678, that encoded a hypothetical protein, observed to be significantly down-regulated by 5.9-fold, was found to resided within a predicted operon containing a gene putatively encoding a Fe (II) pyochelin siderophore transport protein. Possibly in connection with the biosynthesis of pyochelin, a phenazine biosynthesis protein A/B gene (B1568) was observed to be significantly down-regulated (-1.9-fold) in the adapted derivative.

The five outer membrane protein genes observed to be significantly down regulated ≥1.5-fold in the preservative-adapted strain (Table 28), encoded putative porins of a predicted similar size to those that had been up-regulated, ranging from 37.5 to 41.8 kDa. The largest fold-change in expression of 4.1-fold, was observed for the putative 39.8 kDa porin protein gene A3634.

Other significantly down-regulated genes of note included gene A6361 which encoded a putative flagellar hook-associated protein FlgK (down-regulated 1.8-fold, Table 28). This gene was also observed to be down-regulated in the parental strain in response to DMDM hydantoin (Table 24). The expression of other genes within the putative gene cluster A6360 to A6364, that putatively encode structural components that contribute to the assembly of a single flagellar system (orthologous to the gene cluster BCAL0561 to BCAL0577 in *B. cenocepacia* J2315 (Holden *et al.*, 2009)), was observed not to have significantly altered greater than1.5-fold in the adapted derivative.

Table 27. Up-regulated gene expression in preservative-adapted *B. lata* strain 383-CMIT

Gene Name	Fold change	p-value	Putative gene function
A3512 [a TnΔ]	1.7	0.00613	ABC transporter, ATPase subunit
A3514 [a TnΔ]	2.0	0.00105	ABC transporter, inner membrane subunit
A3515 [a]	1.6	0.00904	VacJ-like lipoprotein
A3516 [a]	1.9	0.00151	Toluene tolerance
A3517 [a TnΔ]	2.1	0.000163	hypothetical protein
A3699	1.8	0.00213	transcriptional regulator, AraC family
A3783	1.6	0.000106	Glutamate synthase (NADPH)
A4033	1.5	0.00643	hypothetical protein
A4148	2.7	0.00197	hypothetical protein
A4203	1.8	0.00412	fumarate hydratase
A4255	1.6	0.00129	Excinuclease ABC, C subunit
A4268	1.7	6.69E-05	Electron transport protein SCO1/SenC
A4500	1.6	0.00152	outer membrane protein, (porin)
A4671	1.6	0.00456	Host factor Hfq
A4811	1.9	3.73E-05	Low molecular weight phosphotyrosine protein phosphatase
A4968	2.3	0.0455	Major facilitator superfamily (MFS_1) transporter
A5145	2.9	0.00586	Short-chain dehydrogenase/reductase SDR
A5209 [b]	1.6	0.000151	hypothetical protein
A5210 [b]	1.6	0.00247	protein-methionine-S-oxide reductase
A5212	1.5	0.000504	Transcriptional regulator, BolA
A5373 [c]	2.5	0.000488	Cytochrome C oxidase subunit IV
A5374 [c]	1.7	0.0101	Heme/copper-type cytochrome/quinol oxidase subunit 3-like
A5375 [c]	1.6	0.00182	Cytochrome-c oxidase
A5376 [c]	1.9	0.00124	Cytochrome o ubiquinol oxidase subunit II
A5606	1.7	0.000364	globin-like protein
A5677	1.7	0.00408	transaldolase
A5701	1.5	0.0303	hypothetical protein
A5849	4.7	0.00161	Amino acid tranporter
A5867	1.6	0.00167	Drug resistance transporter EmrB/QacA subfamily
A5874	2.6	6.45E-05	Superoxide dismutase
A5884	1.8	0.000274	hypothetical protein
A5900	1.5	5.48E-05	pyridoxamine 5'-phosphate oxidase
A5963	1.7	0.000401	hypothetical protein
A6000	1.5	0.00253	hypothetical protein
A6017	1.6	0.0039	hypothetical protein
A6115	1.5	0.00262	Sodium/hydrogen exchanger
A6170	1.5	0.0018	hypothetical protein
A6261	2.1	0.000106	hypothetical protein
A6482	2.4	1.32E-05	Transcriptional regulator, LysR family
A6484 [d]	1.8	8.38E-07	Patatin
A6485 [d]	2.8	0.000303	Phenylacetic acid degradation-related protein
B0083	1.9	0.0304	hypothetical protein

Footnotes: All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a greater than two-fold change in expression are shown in bold; [TnΔ] Transposon mutants (9:18:A7, 11:E4, 21:A6 and 30:H9) , interrupted in genes within the operon A3512_3517, demonstrated altered susceptibility to isothiazolone preservatives, as discussed in chapter 5; [a-d] genes within a predicted operon.

Table 27. Continued

Gene Name	Fold change	p-value	Putative gene function
B0084	1.7	0.0219	hypothetical protein
B0088	1.6	0.000502	hypothetical protein
B0105 [e]	1.9	0.00128	Radical SAM oxidoreductase
B0106 [e]	1.9	0.000653	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
B0192	1.6	0.000182	membrane protein
B0213	1.6	0.00241	Aminoglycoside/hydroxyurea antibiotic resistance kinase
B0233	1.7	0.000411	hypothetical protein
B0269 [f]	2.1	0.00705	stress protein
B0270 [f]	1.9	0.0196	stress protein
B0278	1.5	0.000449	hypothetical protein
B0341	1.5	0.000418	transcriptional regulator, AraC family
B0355	1.9	0.0247	Major facilitator superfamily (MFS_1) transporter
B0371	2.3	0.00379	Peptidase S10, serine carboxypeptidase
B0372	1.6	0.000677	NAD(+) synthetase
B0374	1.7	0.000124	hypothetical protein
B0443	3.5	2.84E-05	hypothetical protein
B0522	1.6	0.0153	transcriptional regulator, LysR family
B0540 [g]	2.1	0.00016	ABC transporter, inner membrane subunit
B0541 [g]	2.3	5.44E-06	ABC transporter, ATPase subunit
B0619	2.4	0.0279	hypothetical protein
B0791	1.9	0.00149	protein of unknown function DUF81
B1004 [h]	25.2	0.00038	Secretion protein, HlyD family
B1005 [h]	9.3	0.000108	Hydrophobe/amphiphile efflux pump, HAE1 family
B1006 [h]	10.0	0.0039	RND efflux system, outer membrane lipoprotein, NodT family
B1023	1.8	0.00189	outer membrane protein (porin)
B1025	1.7	0.0212	hypothetical protein
B1059	1.7	0.000352	Metallophosphoesterase
B1260	1.5	0.000456	hypothetical protein
B1270	1.7	0.0145	putative threonine efflux protein-like
B1327	4.6	0.000749	Major facilitator superfamily (MFS_1) transporter
B1331	1.5	0.00389	hypothetical protein
B1355	2.8	0.000485	hypothetical protein
B1467	5.2	0.000879	Carbonic anhydrase/acetyltransferase isoleucine patch superfamily
B1677	1.6	0.0381	hypothetical protein
B1715	2.3	0.000197	TrkA-N, putative potassium channel protein
B1733	1.7	0.00102	short chain dehydrogenase
B1758	1.7	0.00016	ABC nitrate/sulfonate/bicarbonate family transporter, inner membrane subunit
B1768	4.5	0.0054	ABC Fe3+ siderophore transporter, inner membrane subunit
B1770	1.9	0.00771	outer membrane protein (porin)
B1798	1.5	4.13E-05	DNA polymerase IV(family X)
B1906	1.6	0.0009	hypothetical protein

Footnotes: All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a greater than two-fold change in expression are shown in bold ; [e-h] genes within a predicted operon.

Table 27. Continued

Gene Name	Fold change	p-value	Putative gene function
B1910 [i]	1.9	7.58E-05	CDP-alcohol phosphatidyltransferase
B1911 [i]	1.7	0.00269	2OG-Fe(II) oxygenase
B1912 [i]	1.6	0.000123	hypothetical protein
B1913 [j TnΔ]	2.1	4.62E-05	sugar nucleotidyltransferase-like
B1915 [j]	1.6	0.00111	Thiamine pyrophosphate enzyme, putative 3-phosphonopyruvate decarboxylase
B1916 [j]	2.0	8.56E-06	Aminotransferase, class V
B1918	2.2	0.00147	3-oxoacyl-(acyl carrier protein) synthase
B1919	1.6	0.00114	hypothetical protein
B1929	1.7	0.000885	Peptidase S33, proline iminopeptidase 1
B2147	2.2	0.000504	citrate synthase
B2149	1.6	0.0127	succinate dehydrogenase catalytic subunit
B2154	1.6	0.0263	malate dehydrogenase
B2180	1.6	0.000877	hypothetical protein
B2185	2.8	0.00248	hypothetical protein
B2186	1.9	0.00118	GCN5-related N-acetyltransferase
B2211 [k]	1.6	0.0018	1-deoxy-D-xylulose-5-phosphate synthase
B2212 [k]	1.9	0.000803	farnesyl-diphosphate synthase
B2213 [k]	1.8	0.00127	exodeoxyribonuclease VII small subunit
B2300	1.6	0.00035	riboflavin synthase subunit alpha
B2511	1.5	0.00022	GCN5-related N-acetyltransferase
B2598	2.1	0.0488	Peptidase M20D, amidohydrolase
B2902	1.8	0.00143	Acid phosphatase
B2908	1.6	0.0127	hypothetical protein
B2947	1.5	0.000108	Histone deacetylase superfamily
B3063	2.0	0.0134	Glucose-methanol-choline oxidoreductase
B3083	1.5	5.29E-05	PpiC-type peptidyl-prolyl cis-trans isomerase
BR000	2.5	0.0329	(ribosomal)
C6626	1.8	0.000465	Toluene tolerance protein (Ttg2D)
C6627	1.6	0.00105	Transcriptional regulator, LysR
C7150	1.8	0.0214	hypothetical protein
C7246	1.6	0.00277	Glutathione peroxidase
C7294	5.1	0.00205	AMP-dependent synthetase and ligase
C7344	1.9	0.00139	Cytochrome c, class I
C7345	1.9	5.13E-05	Glucose-methanol-choline oxidoreductase
C7346	3.3	0.000394	hypothetical protein
C7469	1.8	0.0171	Lysine exporter family protein (LYSE/YGGA)
C7477	1.6	0.000784	hypothetical protein
C7479	1.5	7.96E-05	Alpha/beta hydrolase
C7497	1.8	0.0176	metal-dependent phosphohydrolase
C7504	2.1	1.29E-05	ABC transporter, inner membrane subunit
C7542	1.7	0.000189	hypothetical protein
C7543	1.9	0.00144	hypothetical protein
CR008	2.7	0.0179	(ribosomal)

Footnotes: All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a greater than two-fold change in expression are shown in bold; [TnΔ] Transposon mutant (29:G3) , interrupted in gene B1913, demonstrated an increased susceptibility in MIT/CMIT screens, as discussed in Chapter 5; [i-k] genes within a predicted operon.

Table 28. Down-regulated gene expression in preservative-adapted *B. lata* strain 383-CMIT

Gene Name	Fold change	p-value	Putative gene function
A3313	-1.6	0.0366	Pyridoxamine 5'-phosphate oxidase-related, FMN-binding
A3369	-2.0	0.0122	protein of unknown function DUF805
A3544	-3.4	0.0384	hypothetical protein
A3545	-1.5	0.0496	hypothetical protein
A3558 [a]	-1.6	0.00514	TPR repeat protein
A3560 [a]	-1.7	0.00366	protein of unknown function DUF877
A3561 [a]	-1.6	0.00354	protein of unknown function DUF796
A3562 [a]	-1.6	0.000772	protein of unknown function DUF1316
A3566 [b]	-1.7	0.016	ImpA-like (Type VI secretion associated)
A3567 [b]	-1.8	9.53E-05	Rhs element Vgr protein
A3568 [b]	-1.9	0.0107	hypothetical protein
A3576	-1.5	0.00809	hypothetical protein
A3634	-4.1	0.000367	outer membrane protein, (porin)
A3758	-1.5	0.00066	ABC amino acid transporter, ATPase subunit
A3863	-1.6	0.00465	Glycosyltransferase-like
A3865	-1.7	0.00912	hypothetical protein
A3905	-2.3	0.00522	Peroxidase
A3918	-1.7	0.00682	D-lactate dehydrogenase
A3949	-4.5	0.00248	ABC Fe3+ transporter, inner membrane subunit
A4113	-3.0	0.00065	cold-shock DNA-binding domain protein
A4383	-1.5	0.0118	cytosine/purines, uracil, thiamine, allantoin transporter
A4384	-1.5	0.0196	outer membrane protein, (porin)
A4521	-1.8	0.0383	dual specificity protein phosphatase
A4704	-2.0	0.00403	alkanesulfonate monooxygenase
A4739	-1.5	0.00944	Thiosulphate-binding protein
A4920	-1.6	0.0074	Methylated-DNA-(protein)-cysteine S-methyltransferase
A4989	-2.4	0.00444	hypothetical protein. Putative transporter
A4990	-1.7	0.00636	hypothetical protein
A4991	-2.0	0.033	3-oxoacyl-(acyl-carrier protein) synthase
A4992	-1.8	0.0219	hypothetical protein
A4993	-1.8	0.0288	Glycine C-acetyltransferase
A4994	-2.1	0.0182	hypothetical protein
A5004	-1.8	0.0384	hypothetical protein
A5274	-1.7	0.0132	transcriptional regulator, LysR family
A5288	-2.0	0.0203	4Fe-4S ferredoxin, iron-sulfur binding
A5507	-1.6	0.00318	Toxic anion resistance
A5803 [TnΔ]	-1.5	0.00698	sulfate adenylyltransferase subunit 2
A5806	-1.5	0.0013	Nitrite/sulfite reductase
A6297	-1.6	0.000471	ABC thiosulphate transporter, periplasmic ligand binding protein
A6361	-1.7	0.0255	flagellar hook-associated protein
B0296	-1.8	0.0315	Hydrophobe/amphiphile efflux pump, HAE1
B0668 [c]	-6.3	0.00659	chorismate mutase
B0670 [c]	-4.3	0.0288	AMP-dependent synthetase and ligase
B0672 [d]	-2.1	0.0184	Non-ribosomal peptide synthetase modules
B0676 [d]	-2.5	0.0176	ABC efflux pump, fused inner membrane and ATPase subunits

Footnotes: All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a greater than two-fold change in expression are shown in bold; [TnΔ] Transposon mutant (27:H4), interrupted in gene A5803, demonstrated an increased susceptibility in the preservative screens, as discussed in Chapter 5; [a-d] genes within a predicted operon.

Table 28. Continued

Gene Name	Fold change	p-value	Putative gene function
B0678	-5.9	0.0476	hypothetical protein [1]
B0681	-4.0	0.0172	transcriptional regulator, AraC family
B0683	-3.1	0.00297	Beta-lactamase
B0686	-2.4	0.014	TonB-dependent receptor
B0811	-1.6	0.0207	GCN5-related N-acetyltransferase
B0937	-3.2	0.0436	transcriptional regulator, AraC family
B0997	-1.5	2.87E-05	Sodium/dicarboxylate symporter
B1428	-1.8	0.0178	hypothetical protein
B1429	-3.1	0.0458	hypothetical protein
B1485	-1.5	0.0233	TonB-dependent receptor [1]
B1496	-1.7	0.0154	FAD dependent oxidoreductase
B1497	-1.9	0.00896	hypothetical protein
B1567	-1.6	0.0238	Acyl-CoA dehydrogenase-like
B1568	-1.9	0.00821	Phenazine biosynthesis protein A/B
B1748	-2.5	6.02E-05	outer membrane protein (porin)
B1771	-1.7	0.00804	Zinc-containing alcohol dehydrogenase superfamily
B1832	-1.5	0.00548	hypothetical protein
B1980	-1.5	0.00318	Na+/solute symporter
B2103	-3.2	0.00737	outer membrane protein (porin)
B2324 [e]	-1.5	0.0169	Aromatic-ring-hydroxylating dioxygenase, beta subunit
B2325 [e]	-2.4	0.00264	ring hydroxylating dioxygenase, alpha subunit/Rieske (2Fe-2S) protein
B2365	-1.5	0.000914	Polyhydroxyalkanoate depolymerase
B2842	-1.6	0.0261	Beta-lactamase-like
B2884	-1.9	0.0143	hypothetical protein
B2926	-1.7	0.0296	hypothetical protein
B2933	-1.6	0.0269	hypothetical protein
B2934	-1.6	0.0121	hypothetical protein
B2939	-1.5	0.0287	outer membrane autotransporter barrel
B3091	-1.5	0.0211	Alpha/beta hydrolase
C6545	-1.6	0.0369	hypothetical protein
C6591	-1.6	0.00379	Haemaglutinin/autotransporter like protein
C6622	-1.7	0.0228	transcriptional regulator, LysR family
C6820	-2.1	0.0194	hypothetical protein
C6876	-1.7	0.00584	collagenase
C7073	-2.6	0.0121	ring hydroxylating dioxygenase, alpha subunit
C7095	-1.6	0.0143	hypothetical protein
C7132	-1.6	0.00398	Short-chain dehydrogenase/reductase SDR
C7142	-2.0	0.0377	FAD dependent oxidoreductase
C7206	-1.5	0.00191	Flp/Fap pilin component
C7251	-1.5	0.0248	Sodium-dicarboxylate symporter
C7252	-1.6	0.0208	hypothetical protein
C7455	-1.7	0.0107	outer membrane protein (porin)
C7548	-3.6	0.0197	Amino acid transporter
C7559	-1.7	0.03	Acetyl-CoA C-acyltransferase
C7691	-2.2	0.00415	protein of unknown function DUF6, transmembrane

Footnotes: All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a greater than two-fold change in expression are shown in bold; [e] genes within a predicted operon; [1] siderophore-iron transport associated.

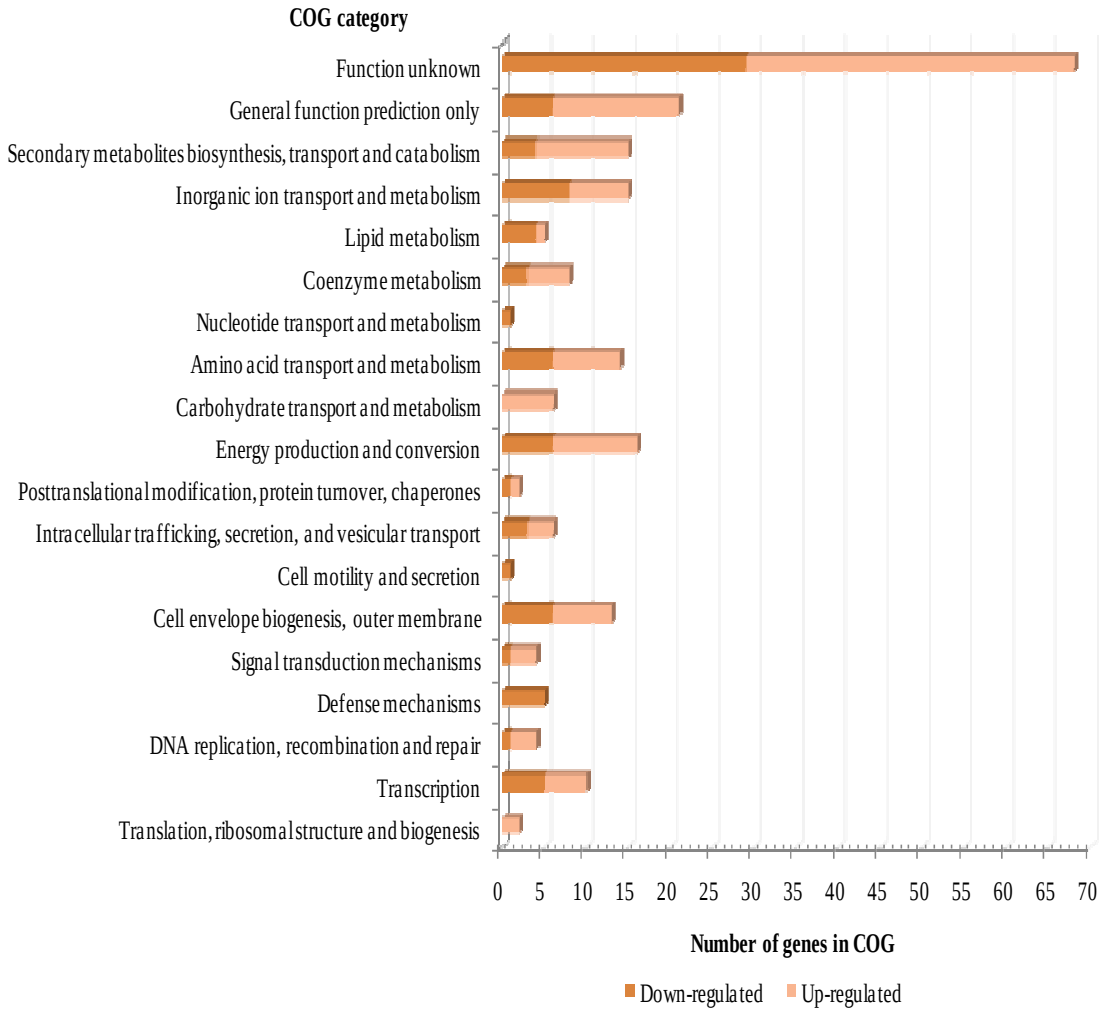
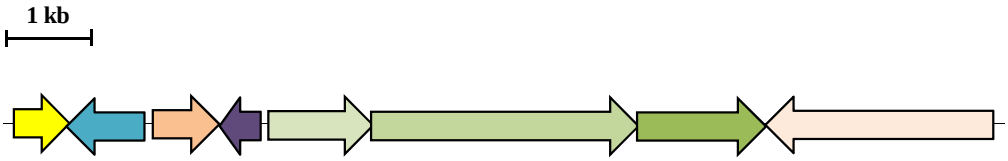


Figure 26. The distribution of differentially expressed genes of preservative-adapted *B. lata* strain 383-CMIT genes, among COG categories.

The dark orange and light orange bars correspond to 216 genes whose expression significantly decreased or increased (respectively) by at least 1.5 fold ($P < 0.05$), relative to expression levels of the parental strain.



Gene	B1000	B1001	B1002	B1003	B1004	B1005	B1006	B1007
Product	TetR	LysR	SDR	MerR	HlyD secretion protein	RND Efflux hydrophobe/ amphiphile protein	RND outer membrane lipoprotein	2-oxoacid dehydrogenase
Size	23 kDa	33 kDa	26 kDa	18 kDa	44 kDa	113 kDa	53 kDa	99 kDa
Localisation	C	U*	U*	U	CM	CM	OM	C
Putative Ortholog	N/A	BCAM 1950	BCAM 1949	BCAM 1948	BCAM 1947	BCAM 1946	BCAM 1945	BCAM 1944
Microarray fold change in expression	NS	NS	NS	+1.4	**+25**	**+9.3**	**+10**	NS

Figure 27. The organisation of genes encoding an RND-efflux system, over expressed in the preservative-adapted *B. lata* 383-CMIT derivative.

The schematic shows the arrangement of three genes (loci B1004_1006, as green arrows), within a putative operon located on the second *B. lata* strain 383 chromosome, that encode proteins of a tripartite efflux system of the RND family; each drawn to the scale given by the bar on the left. The putative gene products, subcellular localisation and putative orthologs in *B. cenocepacia* J2315, are also shown. Abbreviations: C, cytoplasm; CM, cytoplasmic membrane; OM, outer membrane; U, localisation unknown; U*, protein may have multiple localisation sites. The fold change in gene expression in the preservative-adapted derivative compared to the parental *B. lata* strain 383 (based on microarray analysis) are shown; NS: no significant change in expression was observed between the preservative-adapted derivative and the parental strain ($P > 0.05$).

6.2.7 Differential gene expression in the preservative-adapted *B. lata* strain 383-CMIT derivative exposed to MIT/CMIT

Microarray analysis of the preservative-adapted *B. lata* strain 383-CMIT derivative cultured in 0.00001498% MIT/CMIT, demonstrated that 274 coding genes were differentially expressed with a 1.5-fold filter applied; this represented 3.5 % of all CDSs annotated in the *B. lata* strain 383 genome. Filtering with a confidence level of $P<0.05$, the list was reduced to 29 genes: 18 significantly up-regulated (Table 29) and 11 significantly down-regulated ≥1.5 fold (Table 30). Within this list, 11 genes were significantly up-regulated ≥2-fold and three were significantly down-regulated ≥2-fold. Overall, global gene expression did not significantly alter more than 4.2-fold in response to exposure to the priming preservative MIT/CMIT.

The differentially expressed genes were disseminated throughout the *B. lata* strain 383 genome: with 6, 15 and 8 genes located respectively on the first, second and third chromosome. The distribution of the 29 genes with significant differential expression among COG categories is shown in Figure 28. Genes were associated with 15 COG categories involved in metabolism (n= 12), cellular processes and signalling (n= 6), information storage and processing (n= 2), or were poorly characterised in function (n= 9).

An acetylglutamate kinase-like gene (B2621), putatively involved in the biosynthesis of arginine, demonstrated the largest fold-change (+4.2-fold) in expression in response to exposure to sub-inhibitory concentrations of the isothiazolone blend of preservatives MIT/CMIT (Table 29). Arginine is a potential precursor of polyamines and is involved in a variety of degradative pathways (Cunin *et al.*, 1986). In connection, an ABC-type transporter protein of spermidine/putrescine polyamines (gene C6818) was also observed to be significantly down-regulated by 1.7-fold.

A putative homologue of a paraquat-inducible protein A (gene C6818) of unknown function, was observed to be significantly up-regulated (1.5-fold) in response to the isothiazolone preservatives. The paraquat-inducible protein B (gene C6816) within this putative operon, with a predicted extracellular localisation (Winsor, 2008), was observed to be significantly down-regulated 3.7-fold (Tables 29 and 30).

Of the 29 genes observed to have altered significantly in response to the isothiazolone blend, two were putatively associated with motility and adherence. Expression of a secretin assembly protein of a type-IV pili (*Flp;* C7201)(Kachlany *et al.*, 2001) was found to be significantly up-regulated 2.8-fold (Table 29). A structural flagellin protein (*Flg*; A3343) was observed to be significantly down-regulated 1.7-fold in response to the isothiazolone blend (Table 29).

Expression of two outer membrane porins, not observed to have been differentially expressed in the absence of the priming preservative, was observed to have significantly altered in response to the isothiazolone preservatives. These two porins were of a similar predicted size at 38 and 40 kDa; however, gene B1722 was observed to be up-regulated by 2.1-fold (Table 29), whereas gene B0225 was observed to be down-regulated 2.1-fold (Table 30).

Table 29. Up-regulated gene expression of preservative-adapted *B. lata* strain 383-CMIT exposed to the isothiazolone blend MIT/CMIT

Gene Name	Fold change	p-value	Putative gene function
A3269	3.2	0.00729	ABC branched-chain amino acid family transporter, inner membrane subunit
A4539	2.4	0.00947	metal-dependent hydrolase
A5942	2.0	0.0135	Isochorismatase hydrolase
B0175	1.7	0.0477	Cytochrome bd ubiquinol oxidase, subunit I
B0937	2.1	0.0325	transcriptional regulator, AraC family
B0950	1.7	0.0465	Major facilitator superfamily (MFS_1) transporter
B1443	1.8	0.0349	short chain dehydrogenase
B1665	1.6	0.0337	transcriptional regulator, Crp/Fnr family
B1722	2.1	0.0199	outer membrane protein (porin)
B2299	2.2	0.0491	ABC efflux pump, fused ATPase and inner membrane subunits
B2475	1.7	0.0103	Amidohydrolase
B2621	4.2	0.0135	Acetylglutamate kinase-like
B2638	2.3	0.00205	hypothetical protein
C6717	1.7	0.0389	ABC spermidine/putrescine transporter, inner membrane subunit
C6818	1.5	0.0369	Paraquat-inducible protein A
C7123	2.4	0.015	Short-chain dehydrogenase/reductase SDR
C7201	2.8	0.0367	Flp pilus assembly protein secretin CpaC
C7402	2.2	0.00971	hypothetical protein

Footnotes: All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a greater than two-fold change in expression are shown in bold

Table 30. Down-regulated gene expression of preservative-adapted *B. lata* strain 383-CMIT exposed to the isothiazolone blend MIT/CMIT

Gene Name	Fold change	p-value	Putative gene function
A3343	-1.7	0.0387	Flagellin
A4309	-1.6	0.0361	phosphatase-like
A5858	-1.6	0.0489	hypothetical protein
B0225	-2.1	0.0156	outer membrane protein (porin)
B1961	-1.6	0.0067	glutamine ABC transporter periplasmic-binding protein
B2852	-1.5	0.0428	Alkaline phosphatase
B3016	-3.0	0.00317	urocanate hydratase
BR000	-1.9	0.0459	16S rRNA
C6816	-3.7	0.0189	Paraquat-inducible protein B'
C7537	-1.5	0.0202	hypothetical protein
C7633	-1.6	0.0213	Pirin-like protein

Footnotes: All genes shown were significantly altered in expression by ≥1.5-fold ($p<0.05$) and genes with a greater than two-fold change in expression are shown in bold

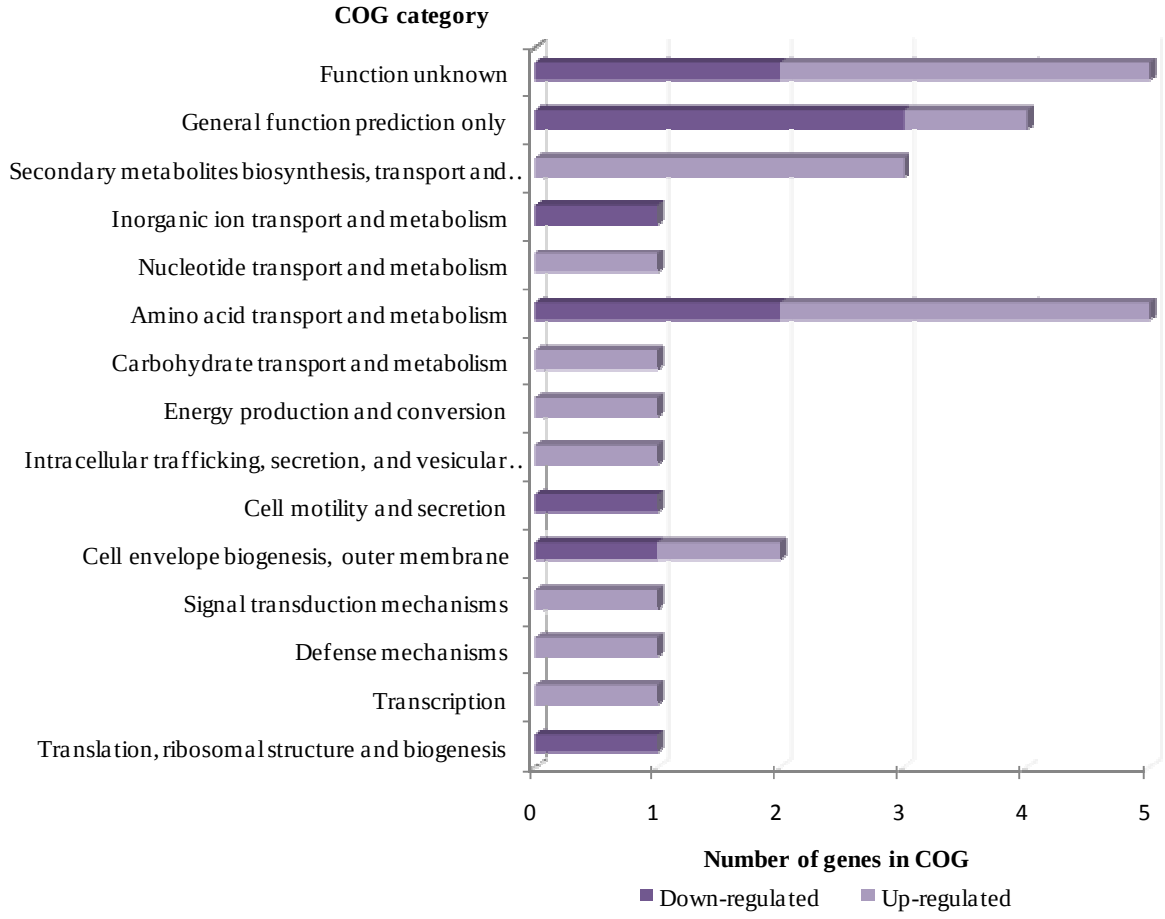


Figure 28. The distribution of differentially expressed genes of preservative-adapted *B. lata* strain 383-CMIT genes in response to MIT/CMIT exposure, among COG categories.

The dark and light purple bars correspond to 28 genes whose expression significantly decreased or increased (respectively), by at least 1.5 fold ($P < 0.05$), relative to the expression levels of the preservative-adapted derivative cultured in a medium containing 0.00001498% v/v MIT/CMIT.

6.2.8 Expression analysis of the RND efflux pump gene B1004 by qRT-PCR

Global gene expression analysis of the preservative-adapted *B. lata* strain 383-CMIT, revealed an over-expression of a tripartite RND-type efflux system (loci B1004 to B1006), irrespective of the presence of the priming preservative MIT/CMIT (section 6.2.6). Subsequently, gene expression of the secretion protein component (gene B1004) of the efflux system (Figure 27), in *B. lata* strain 383 and the preservative-adapted *B. lata* strain 383-CMIT derivative, was analysed using qRT-PCR as described in section 2.8 To extend our gene expression analysis, we evaluated whether the RND-efflux gene was up-regulated in three *B. lata* isolates (Bcc1294, Bcc1296, and Bcc1406) from environmental-industrial sources when cultured in the absence of preservatives. *B. lata* strain Bcc1296 was selected for analysis based on a high native tolerance to the isothiazolone blend MIT/CMIT that had been observed in the survey of preservative susceptibility (Chapter 3). The MIC value of MIT/CMIT for this strain (0.000674% v/v) was found to be nine-fold higher than that of the reference *B. lata* strain 383 (0.0000749% v/v), and the other *B. lata* strains isolated from environmental-industrial sources (Chapter 3).

qRT-PCR analysis of the isothiazolone-adapted *B. lata* strain 383 derivatives, revealed that changes in the relative expression of gene B1004 (from that of *B. lata* strain 383) were largest for derivatives *B. lata* strain 383-CMIT (116.8 ± 62.4 fold) and *B. lata* strain 383-BIT (81 ± 20.2 fold) (Table 31). Only a minor increase in gene B1004 expression (1.6 ± 1.5 fold) was observed for the methylisothiazolinone adapted derivative *B. lata* strain 383-MIT (Table 31). For *B. lata* strains isolated from an environmental-industrial source, the largest change in relative expression of the RND-efflux gene was observed in a *B. lata* strain Bcc1294, with a fold-change of 236.8 (± 184.4) from transcription levels in *B. lata* strain 383 (Table 31). The two other *B. lata* isolates from environmental-industrial sources demonstrated minor changes in the relative expression of the efflux gene. Transcription in strain Bcc1396 was 5.7 (± 1) fold higher than *B. lata* strain 383, while transcription in strain Bcc1406 was found to be 3.3 (± 2.4) fold higher than *B. lata* strain 383 (Table 31).

Table 31. Relative expression levels of RND efflux gene in *B. lata* isolates and preservative-adapted *B. lata* strain 383 derivatives, as determined by qRT-PCR

	Mean relative expression in *B. lata* strain *(* ±STDEV*)*						
Gene	383 *wt*	383-CMIT	383-MIT	383-BIT	1294	1296	1406
B1004	1 (0)	**116.8** (62.4)	1.6 (1.5)	**81.0** (20.2)	**236.8** (184.4)	**5.7** (1)	**3.3** (2.4)

Footnotes:

Values represent fold change (mean of biological duplicates) in comparison to the transcription level in *B. lata* strain 383. Data are normalised to the expression level of the reference gene *phaC* (A5090) producing a lower fold change than when normalised to the *recA* gene in .

Values ≥ 2 fold change are shown in bold

qRT-PCR, quantitative real-time polymerase chain reaction

6.2.9 The affect of efflux inhibition on the isothiazolone and ciprofloxacin susceptibility of *B. lata* strain 383 and preservative-adapted derivatives

Antibiotic susceptibility profiling of the preservative-adapted *B. lata* strain 383 derivatives demonstrated that in addition to a decreased susceptibility to isothiazolone preservatives, *B. lata* strain 383-CMIT and 383-BIT displayed decreased susceptibility to fluoroquinolone antibiotics (Chapter 4). Global gene expression analysis of the adapted derivative *B. lata* strain 383-CMIT revealed the over-expression of the B1004-B1006 encoded RND-type efflux system, irrespective of the presence of the priming preservative MIT/CMIT (Table 27, section 6.2.6). Microarray data were subsequently corroborated by gene expression analysis of the RND-efflux gene B1004, using quantitative RT-PCR (section 6.2.3).

In addition to target modification, active efflux has been attributed to increased fluoroquinolone resistance in Gram-negative bacteria (Drlica & Malik, 2003). To assess the role of RND-efflux in the susceptibility of *B. lata* strain 383 to the isothiazolone preservative blend and ciprofloxacin, efflux inhibition assays were performed using the efflux inhibitor MC-207,110 (PAβN), as described in section 2.16. The dipeptide compound has been shown effectively to inhibit clinically relevant RND-efflux pumps in Gram-negative bacteria by working as an active competitor of multiple structurally dissimilar antibiotics (Kvist *et al.*, 2008; Pagès *et al.*, 2005).

The sensitivity of the parental *B. lata* strain 383, and its preservative-adapted derivative *B. lata* strain 38-CMIT, to the isothiazolone preservative blend MIT/CMIT and ciprofloxacin, increased in the presence of 0.512 µg/ml of the efflux inhibitor (Table 32). The parental *B. lata* strain demonstrated a 2-fold and 3-fold reduction respectively, in MIC values for ciprofloxacin (1.41 ± 0.6 µg/ml) and MIT/CMIT (3.49E-0.5 µg/ml) (Table 32). The preservative-adapted derivative demonstrated a 6-fold and 4-fold reduction respectively in MIC values for ciprofloxacin (11.25 ± 5.3 µg/ml) and MIT/CMIT (2.81E-04 ± 1.32E-04%) (Table 32). Although a greater fold-change in MIC values were observed for the preservative-adapted derivative, its ciprofloxacin and MIT/CMIT susceptibility was not reduced to wild-type levels in the presence of the efflux inhibitor (Table 32).

Table 32. The MICs of ciprofloxacin and the MIT/CMIT isothiazolone blend in the presence and absence of the efflux pump inhibitor MC-207,110

Strain	Ciprofloxacin MIC (µg/ml)		Fold change	Isothiazolone blend MIC (%)		Fold change
	CIP alone	CIP plus 512 mg/L MC-207,110		MIT/CMIT alone	MIT/CMIT plus 512 mg/L MC-207,110	
B. lata 383	1.41 (±0.6)	0.70 (±0.3)	2	3.49E-05 (±1.63E-05)	1.05E-05 (±0)	3.3
B. lata 383-CMIT	11.25 (±5.3)	1.88 (±0)	6	2.81E-04 (±1.32E-04)	7.02E-05 (±3.31E-05)	4

Footnotes:

Values represent the mean MIC values (± STDEV), and mean fold change, of two biological replicates with a total of 6 technical replicates.

6.3 DISCUSSION

Transcriptomic analysis, using a custom made *B. lata* strain 383 DNA microarray, was successfully used to gain an insight into the response of *B. lata* strain 383 to DMDM hydantoin and the isothiazolone blend of, and the changes in the transcriptome associated with preservative-induced adaptive resistance. Quantitative RT-PCR and semi-quantitative PCR expression analysis of select genes was used to validate the microarray data. Overall, the up- or down-regulation of genes observed by these methods correlated to the microarray results. Global mapping revealed multiple genetic pathways are putatively utilised by *B. lata* strain 383 to resist sub-MIC levels of preservatives, and are altered in the preservative adapted derivative.

The role of these determinants in *B. lata* strain 383 resistance to preservatives remains to be confirmed, via the generation of isogenic knock-out mutants and perhaps proteomic analysis. An inherent limitation of the DNA microarray data is that the resulting transcriptome does not take into account posttranslational events. Although in most cases there is a high correlation between the transcriptome and the proteome (Washburn & Yates Iii, 2000) (Ehrenreich, 2006; Zhang *et al.*, 2010), the production of mRNA does not necessarily predict that a protein will be translated and expressed from a transcript. Conversely, proteomic analysis has been shown to detect differentially expressed proteins that were not detected as differentially expressed in the transcriptome (Zlosnik *et al.*, 2008). This may therefore identify additional resistance determinants that may not have been identified in the present study.

6.3.1 *B. lata* strain 383 global gene expression in the presence of DMDM hydantoin

The transcriptomic microarray-based approach revealed that there were few significant alterations in *B. lata* strain 383 gene expression in response to sub-inhibitory concentrations of the formaldehyde releasing agent DMDM hydantoin. In addition, the observed significant changes in gene expression were small, less than, or equal to, a 5.6-fold change from the transcriptional levels observed in the absence of the preservative. Genes with the largest fold-change were associated with metabolism, amino acid or dipetide transport, and the biosynthesis of the outer membrane. These did not include genes encoding known formaldehyde resistance mechanisms.

Bacterial resistance to formaldehyde and formaldehyde donors such as DMDM hydantoin has been associated with an increased synthesis of formaldehyde dehydrogenases. The

investigation of *B. lata* strain 383 DMDM hydantoin resistance determinants, using a transposon mutagenesis-based approach, had identified a zinc-containing alcohol dehydrogenase , putatively involved in the metabolism of the toxic formaldehyde released from DMDM hydantoin via a glutathione-dependent formaldehyde dehydrogenase pathway (Chapter 5). Interestingly, the transcriptomic analysis of *B. lata* strain 383 global gene expression in the presence of the formaldehyde releasing agent DMDM hydantoin, did not reveal a significant alteration in the expression of this gene or others that are putatively involved in pathways for formaldehyde detoxification. The zinc-containing 'long-chain' alcohol dehydrogenase gene that was observed to have been up-regulated 5.6-fold in response to the formaldehyde releasing agent had poor homology to formaldehyde dehydrogenases identified in *Burkholderia* species, with less than 30% homology to the GSH-formaldehyde dehydrogenase (Bxe_A0713) in *B. xenovorans* (Winsor, 2008). This suggests that either the detoxification of formaldehyde is not a primary *B. lata* strain 383 resistance determinant or that the concentration of DMDM hydantoin evaluated was insufficient to induce a significant change in the expression of genes involved in formaldehyde metabolism. The induction of *E. coli* and *H. influenza* glutathione-dependent formaldehyde dehydrogenases has been shown to occur at concentrations matched to the kinetic properties of the enzyme, with concentrations of formaldehyde less than 0.6 ppm resulting in negligible amounts of induction (Gutheil *et al.*, 1997).

The investigation of resistance determinants using a transposon mutagenesis-based approach also identified a putative type II general secretory pathway (Chapter 5). Transcriptomic analysis revealed that minor significant changes in expression (less than a 1.5-fold decrease) of several genes within this genetic pathway had occurred in *B. lata* strain 383 in response to sub-MIC of DMDM hydantoin. Although only minor changes were observed, the significant decrease in the expression of several secretion system protein genes may have had a dramatic effect on the physiological activity of the secretion system. The correlation of the transposon mutagenesis findings with the transcriptomic analysis of *B. lata* strain 383 would therefore suggest that a reduction in the activity of this secretion system may be associated with elevated tolerance to DMDM hydantoin. Further research is required to validate this assumption, and to explore the putative role of this type II secretion system in *B. lata* strain 383 preservative resistance.

Transcriptional analysis of *B. lata* strain 383 genes down-regulated in response to DMDM hydantoin revealed genes associated with motility and chemotaxis. This included a gene

encoding a putative flagellar hook-associated protein and a homologue of CheA, a histidine kinase signal transducer that may participate in the regulation of chemotaxis (Hess *et al.*, 1988). The transition from motility to sessility is a recognised antimicrobial resistance mechanism. Sessile cells, especially those that are biofilm-associated, are often considered to be less susceptible to antimicrobials than their planktonic counterparts (Donlan & Costerton, 2002). In members of the Bcc, several clusters of discrete genes across the genome are reported to contribute to the synthesis and assembly of the whole flagellum (Holden *et al.*, 2009). On closer inspection, the expression of other flagellar structural genes in *B. lata* strain 383 remained unaffected or had small significant changes of less than 1.3-fold. This would suggest that it is unlikely that a significant alteration to the flagellum occurred in response to sub-MIC of DMDM hydantoin.

6.3.2 *B. lata* strain 383 global gene expression in the presence of the isothiazolone preservative blend MIT/CMIT

The transcriptomic analysis of the global gene expression of *B. lata* strain 383 in response to sub-MIC of the isothiazolone blend of preservatives MIT/CMIT revealed few significant changes in expression greater than 1.5-fold, perhaps as a result of the evaluated preservative concentration being too low to induce major changes in gene expression or because the combined action of numerous smaller fold-changes in gene expression translated into a biologically significant effect on preservative susceptibility.

The exposure of *B. lata* strain 383 to the isothiazolone blend resulted in a larger number of genes being significantly up-regulated. The majority of the genes with altered expression were associated with the biosynthesis of the outer membrane or metabolism. Interestingly, this included the up-regulation of a putative capsule polysaccharide biosynthesis gene. The capsular polysaccharide export protein within the putative cluster of 12 genes involved in the biosynthesis and export of capsule polysaccharide (Winsor, 2008) was also observed to be over expressed. However, this export protein gene was excluded from the final gene list because of the filtering criteria. The bacterial capsule constitutes the outermost layer of the cell, and consists of highly hydrated capsular polysaccharides that are linked to the cell surface via covalent attachments to either phospholipids or lipid-A molecules (Roberts, 1996). The increased expression of capsular polysaccharides may indicate that the formation of a capsule may play a role in the defence of *B. lata* strain 383 against the harmful effects of the preservative agent, either directly or by promoting adherence. Capsular polysaccharides are thought to promote the adherence of bacteria to each other

and to surfaces, thereby facilitating the formation of a biofilm (Roberts, 1996). The putative role of the polysaccharide in *B. lata* preservative resistance may be worthy of further investigation.

The investigation of *B. lata* strain 383 determinants in MIT/CMIT resistance using a transposon mutagenesis-based approach identified several putative genetic pathways that were multifactorial in nature (Chapter 5). This included a putative ABC-type transporter system homologous to the Ttg2 system involved in resistance to organic solvents in *P. aeruginosa* (Ramos *et al.*, 2002). The transcriptomic analysis of *B. lata* strain 383 global gene expression in response to sub-MIC of the isothiazolone blend, did not reveal the induction of this genetic pathway. This may indicate that the transporter is not a primary *B. lata* strain 383 isothiazolone resistance mechanism or that the concentration of preservative evaluated was insufficient to induce its expression.

The expression of a putative phage terminase GpA gene that had also been identified as a putative resistance determinant by transposon mutagenesis, had significantly altered in response to the isothiazolone preservatives. The role of the putative prophage terminase in susceptibility to isothiazolone agents is not clear, as the expression of other phage-related genes within the element (loci B1028_B1047) did not significantly alter by greater than 1.2-fold. This prophage module contains many genes encoding small hypothetical proteins without functional annotations as well as homologues of *Burkholderia* phage proteins such as Bcep22 (a phage whose hosts originate from agricultural soils) and a homologue of a *B. pseudomallei* phage protein gp33. While *Burkholderia* phages are not reported to carry known pathogenicity factors or toxins, some encoded proteins that might contribute to the fitness of the host outside the lytic phage replication cycle (Summer *et al.*, 2007). For example, *B. cenocepacia* phage BcepB1A encodes a homologue of phosphoadenosine phosphosulfate reductase, an enzyme that functions in the assimilatory sulphate reduction pathway. In *Burkholderia*, limited sulphate assimilation has been shown to affect the biosynthesis of the siderophore pyochelin, used to sequester iron from the environment (Farmer & Thomas, 2004).

6.3.3 The global gene expression of the preservative-adapted *B. lata* strain 383CMIT

Transcriptomic analysis of global gene expression, in the preservative-adapted *B. lata* strain 383-CMIT, revealed that changes to the transcriptome were not transient and were stably maintained in the adapted-derivative, in the absence of the priming preservative. The number of differentially expressed genes was considerably higher in the preservative-adapted derivative than in the parental strain, following exposure to sub-MIC concentrations of both DMDM hydantoin and isothiazolone preservatives; and, overall, involved larger-fold changes in expression. In addition, isothiazolone-induced adaptive resistance in *B. lata* strain 383 involved significant changes in the expression of genes that were distinct from those altered by a single sub-MIC exposure. In total, only five genes whose expression was observed to have significantly altered by at least 1.5-fold in the parental strain exposed to sub-MIC of the isothiazolone blend, were observed to have a stable alteration of expression in the preservative-adapted derivative (Figure 29). Genes that were differentially expressed in both the parental and derivative strain were associated with metabolism or the transport of amino acids.

The largest significant alteration in gene expression observed in the preservative-adapted strain was not induced by a single exposure to sub-MIC in the parental strain. The expression of an RND-type efflux system was found to be over expressed (up-regulated by up to 25-fold) in the preservative-adapted derivative irrespective of the presence of the priming preservative. This RND efflux system, located on the second *B. lata* chromosome, was found to be one of the few orthologs of the recently characterised RND-9 operon in *B. cenocepacia* (encoded by BCAM1945-1947 genes) (Guglierame *et al.*, 2006; Perrin *et al.*, 2010). The RND-9 operon shares amino acid similarity to the well characterised multidrug MexEF-OprN efflux system in *P. aeruginosa* (Poole, 2001). Interest in this efflux system has grown since the observed over-expression of the BCAM1947 gene in sputum from CF patients (Drevinek *et al.*, 2008). Recent studies, utilising *rnd* knockout mutants of *B. cenocepacia* J2315 (Buroni *et al.*, 2009; Coenye *et al.*, 2011) have focused on determining the biological significance and role of *B. cenocepacia* RND-efflux systems. A phenotypic study of *rnd* mutants by Coenye *et al.* (2011) demonstrated that RND-9 may be a lifestyle-specific chlorhexidine tolerance mechanism of *B. cenocepacia*, linked to resistance in sessile, but not planktonic, cells. Transcriptomic and phenotypic analysis of *rnd* mutants by Bazzini *et al.* (2011) revealed that the biological role of the RND-9 system may not be

restricted to the sole transport of toxic compounds; rather, it may play an important wider role in motility and/or chemotaxis.

Gene expression analysis by qualitative RT-PCR corroborated the over-expression of the RND-efflux B1004 gene in the *B. lata* strain 383-CMIT derivative, observed in the microarray data. Subsequent gene expression analysis revealed that the RND-efflux gene was also over-expressed in the benzisothiazolinone-adapted *B. lata* strain 383 derivative in the absence of the priming preservative. Interestingly, expression of the RND-efflux gene in the methylisothiazolinone-adapted derivative had not greatly increased from that of the parental strain and was considerably lower than that of the other isothiazolone-adapted derivatives. This suggests that that the RND-efflux system may be an agent-specific preservative resistance mechanism of *B. lata* strain 383. Further investigation is required to confirm this speculation, via the creation of an RND-efflux knockout mutant in *B. lata* strain with subsequent preservative susceptibility profiling, and/or by RND-efflux gene expression analysis of *B. lata* strain 383 derivatives adapted to preservatives of a different class.

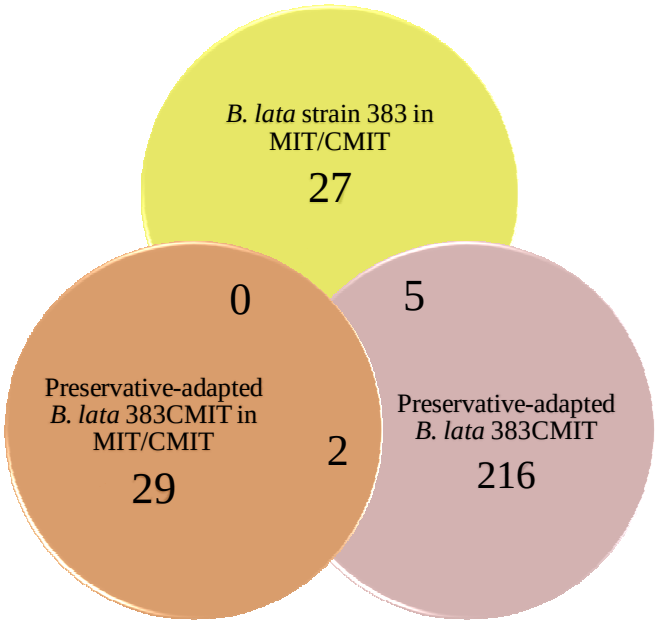


Figure 29. The number of overlapping significantly differentially expressed genes in *B. lata* strain 383 and its preservative-adapted derivative.

The Venn diagram shows the number of genes that were significantly altered in expression (>1.5-fold, $p<0.05$) in *B. lata* strain 383 in response to the isothiazolone blend of preservatives MIT/CMIT; in the preservative-adapted *B. lata* strain 38-CMIT derivative, in the absence of preservative; and in the preservative-adapted *B. lata* strain 38-CMIT derivative in response to the isothiazolone blend MIT/CMIT. Five genes (B0371, B1025, B1771, C7132 and C7548) observed to have a significantly altered in expression in *B. lata* strain 383 in response to the isothiazolone blend were also significantly altered in the preservative-adapted derivative *B. lata* strain 383-CMIT cultured in the absence of preservatives. Two genes (B0937 and BR000) were significantly altered in the preservative-adapted derivative in the absence and presence of preservative; however, the AraC family transcriptional regulator gene B0937, was observed to be down-regulated in the absence but up-regulated in the presence of preservative.

The regulation of RND-efflux systems in Gram-negative bacteria is usually tightly regulated (Grkovic *et al.*, 2002) and many pump component-encoding operons contain a physically linked regulatory gene that encodes either a repressor or an activator protein (Kumar & Schweizer, 2005). In *P. aeruginosa*, the over expression of RND-efflux systems has been attributed to mutation of the regulator protein genes (Schweizer, 2003). In *B. cenocepacia* J2315, a hypothetical MerR family regulatory gene (BCAM1948) of the orthologous RND-9 operon was identified as a putative regulator of the system (Guglierame *et al.*, 2006). The putative regulatory gene is located adjacent to the gene encoding the periplasmic membrane fusion protein (BCAM1947), which is located next to that for the transporter protein (BCAM1946), and is divergently transcribed (Guglierame *et al.*, 2006). The orthologous RND-efflux operon in *B. lata* strain 383, whose orientation is flipped to that of *B. cenocepacia* (Winsor, 2008), also has a divergently transcribed MerR family transcriptional regulator (B1003) located adjacent to the periplasmic membrane fusion protein gene (B1004). The expression of this putative transcriptional regulator gene was observed to have significantly increased by 1.4-fold in the preservative-adapted derivative, but was excluded from the final gene list by filtering criteria. Further investigation is required to confirm the role of this putative regulatory protein in the regulation of the RND-efflux system in *B. lata* strain 383, and re-sequencing is required to determine whether mutation of the regulatory protein gene was associated with the over-expression of the efflux-system in the isothiazolone-adapted derivative.

Efflux inhibition assays demonstrated that the susceptibility of the preservative-adapted derivative *B. lata* strain 383-CMIT, to both the isothiazolone preservative blend MIT/CMIT and ciprofloxacin, increased in the presence of the efflux inhibitor MC-207,110 but failed to revert to wild-type levels. This suggests that either the inhibition of preservative efflux was insufficient, or other mechanisms are significantly contributing to isothiazolone and ciprofloxacin resistance in the preservative-adapted *B. lata* strain 383-CMIT derivative.

Gene expression analysis of *B. lata* isolates from environmental-industrial sources demonstrated higher levels of RND-efflux B1004 gene expression than the reference strain *B. lata* strain 383. This suggests that RND-efflux may also play a role in the native preservative resistance of *B. lata* strains from environmental-industrial sources, and consequently may be a potential target to improve the efficacy of current preservation strategies in industry. The level of relative RND-efflux B1004 expression was observed to

vary between the *B. lata* strains. The *B. lata* strain Bcc1294 demonstrated the highest-fold change in RND-efflux B1004 gene expression, with relative levels considerably higher than the laboratory-derived preservative-adapted *B. lata* strain 383 derivatives. Interestingly, this environmental-industrial isolate was observed to be highly tolerant of isothiazolone preservatives in the survey of Bcc preservative susceptibility (Chapter 3). Further investigation is required to determine whether RND-efflux is a primary native resistance mechanism of this *B. lata* strain, and other native strains that demonstrate elevated isothiazolone tolerance.

The susceptibility of a *B. cenocepacia* J2315 RND-9 knockout mutant (Coenye *et al.*, 2011) to the isothiazolone preservative blend was briefly assessed, by microdilution assay (section 2.16 in Chapter 2), in order to investigate the role of the orthologous RND-efflux system in the preservative resistance of Bcc species other than *B. lata*. The isothiazolone susceptibility of the *B. cenocepacia* RND9-efflux mutant was observed to be the same as the parental strain (data not shown). This suggests that the RND-9 efflux system may not be a primary isothiazolone resistance mechanism in *B. cenocepacia* J2315. However, as the parental strain had a low native tolerance to the isothiazolone blend when evaluated in a BSM (CYG) medium, the baseline level of susceptibility may have prevented the identification of subtle changes in the susceptibility of the *B. cenocepacia* J2315 RND9-efflux mutant.

In addition to the over expression of RND-efflux genes (B1004_B1006), the transcriptomic analysis of global gene expression in the preservative-adapted *B. lata* strain 383-CMIT revealed other putative resistance determinants. These included an observed significant increase in the expression genes encoding a putative ABC-type efflux system, with homology to the Ttg2 transporter being associated with resistance to organic solvents in *P. aeruginosa* (Ramos *et al.*, 2002). This suggested that the efflux of isothiazolone preservatives may involve more than one type of efflux pump system in *B. lata* strain 383. The putative role of this efflux system in *B. lata* strain 383 resistance to isothiazolones was corroborated by its previous identification as a putative isothiazolone resistance determinant in the transposon mutagenesis study (Chapter 5). The baseline expression levels of this efflux-system did not significantly alter in the parental *B. lata* strain 383, in response to sub-MIC of the isothiazolone blend. This would suggest that its induction or over-expression in *B. lata* strain 383 occurs in the presence of higher isothiazolone concentrations. The putative role of the ABC efflux-system in *B. lata* strain 383

isothiazolone resistance requires confirmation. This may be achieved via the generation of an ABC-efflux knockout mutant by methods described by Flannagan *et al*. (2008).

The significant fold-change in the expression of a homologue of the superoxide dismutase SodB, and a minor change in the expression of a homologue of SodC, suggested that the induction of bacterial defence against oxidative stress had occurred as a result of prolonged exposure to increasing sub-lethal concentrations of the isothiazolone preservatives However, the reasons why other antioxidant encoding genes such as catalases remained unaffected, or were down-regulated as in the case of a putative peroxidise, are unknown. Superoxide dismutase detoxifies the O_2^- anion by a dismutation reaction that generates H_2O_2 and O_2 (Fridovich, 1995), thereby preventing the accumulation of the toxic anion that can cause lethal damage to cellular proteins, membranes and nucleic acids (Lefebre & Valvano, 2001). Intracellular reactive oxygen species, such as superoxide (O_2^-), can arise naturally via the metabolism of oxygen and/or from the antimicrobial activity of certain antimicrobial compounds, including the electrophilic isothiazolones. The observed increase in expression of superoxide dismutase in *B. lata* strain 383-CMIT may provide greater protection from oxidative damage, thereby putatively contributing to isothiazolone resistance.

In addition to identifying putative resistance determinants, the transcriptomic analysis also revealed interesting changes in the expression of genes associated with the production of the siderophore pyochelin, as several genes associated with the biosynthesis and transport of the siderophore were down-regulated in the preservative-adapted derivative. In order for Bcc bacteria to survive, iron must be scavenged via the production and uptake of siderophores, which are low molecular weight chelating molecules that sequester iron from the other iron-containing molecules present in the surroundings (Vial *et al.*, 2007). In Bcc bacteria, pyochelin is biosynthesised from salicylate by the successive addition and cyclisation of two molecules of cysteine (Thomas, 2007). The biosynthesis of pyochelin, like other siderophores, is regulated by the availability of iron (Farmer & Thomas, 2004). However, its regulation may also involve the availability of assimilatable sulphur. The biosynthesis of pyochelin in *B. cenocepacia* was found to be particularly sensitive to the availability of assimilable sulphur from the intracellular cysteine pool (Farmer & Thomas, 2004). The observed decrease in expression of genes associated with pyochelin biosynthesis and transport in the preservative-adapted *B. lata* strain 383-CMIT derivative may be indicative of iron limitation. However, it may also be an indication of a depletion

of intracellular cysteine pools via prolonged exposure to isothiazolones which interact with vulnerable protein sulhydryls.

6.3.4 The global gene expression of the preservative-adapted *B. lata* strain 383CMIT in response to MIT/CMIT

The exposure of the preservative-adapted *B. lata* strain 383-CMIT to sub-MIC concentrations of isothiazolone preservatives resulted in few significant changes in gene expression. This is perhaps a result of the evaluated preservative concentration, which was low enough to enable the growth of the wild-type, being insufficient to elicit a dramatic response from the adapted derivative, as it had become accustomed to exposure to higher concentrations. Although the observed changes in gene expression were relatively small (less than 4.2-fold), they involved genes different from those that were observed to have differential expression in the preservative-adapted derivative, and in the parental strain in response to MIT/CMIT. Only two genes in the preservative-adapted derivative were observed to have a significantly altered expression in the presence, and absence, of MIT/CMIT preservatives. These included an AraC family transcriptional regulator, which was found to be significantly down-regulated in the absence of the priming preservative but up-regulated in its presence, and a 16S rRNA gene.

The largest fold-change in gene expression was observed for a putative acetylglutamate kinase-like gene, an enzyme involved in the second step in the route of arginine biosynthesis (Ramón-Maiques *et al.*, 2006). Arginine is synthesised from glutamate through the intermediate generation of ornithine (Cunin *et al.*, 1986). It is an important amino acid involved in various biological processes, and along with ornithine, is a potential precursor of polyamines. The expression of an ABC-type transport protein gene, putatively involved in the transport/uptake of the polyamines spermidine and putrescine (Tabor & Tabor, 1985), was also observed to be significantly up-regulated in response to MIT/CMIT. The increased expression of these genes in the preservative-adapted derivative may be a result of, or a mechanism against the generation of, ROS and/or oxidative stress caused by MIT/CMIT exposure. The activity of enzymes involved in polyamine synthesis, and proteins involved in their transport, have been shown to increase in *E.* coli in response to oxidative stress (Tkachenko & Nesterova, 2003). Polyamines are shown to inhibit the opening of porins in the outer membrane and a consequent decrease in the permeability of the outer membrane has been linked to increased polyamine synthesis and export (Nikaido,

2003). The reduced permeability potentially limits uptake of exogenous sources of ROS, thereby promoting defence against damage induced by oxidative stress.

In addition to the above, significant changes in the expression of putative paraquat-inducible protein genes with unknown function, were observed in *B. lata* strain 383-CMIT. As paraquat is a superoxide radical-generating agent, this would suggest that exposure to sub-MIC of the isothiazolone preservative may have generated superoxide radicals and consequently the induction of defence mechanisms against damage induced by oxidative stress. Why the expression of these paraquat-inducible proteins was not altered in the parental *B. lata* strain 383, when subjected to the same sub-MIC as its preservative-adapted derivative, is unknown.

Trancriptomic analysis also revealed the down-regulation of a homologue of flagellin gene (*fliC*) in the preservative-adapted derivative, in response to sub-MIC of MIT/CMIT. The expression of other genes encoding flagellum structural components, with the exception of gene A3342, remained unaffected in the presence of sub-MIC of the isothiazolone blend. The expression of gene A3342, encoding a putative flagellar hook protein, was also observed to be down-regulated 1.5-fold but was excluded from the list by filtering criteria. The down-regulation of only two out of several genes that contribute to the assembly of the whole flagellum is insufficient evidence to suggest a decrease in the motility of *B. lata* strain 383-CMIT in response to sub-MIC of the priming preservative. However, changes in the expression of *B. lata* strain 383 appendages were not limited to the putative flagellum genes, but also occurred in a putative pili-encoding gene. The gene for a structural unit of a putatively flp-type IV pili (Kachlany *et al.*, 2001) involved in non-specific adherence to surfaces, was observed to be up-regulated 2.8-fold in response to MIT/CMIT preservatives. The combined observations may indicate a decrease in motility and potentially the promotion of adherence to surfaces (i.e. a transition from motile to sessile state) of the preservative-adapted derivative in the presence of sub-MIC of the isothiazolone preservative blend; however this is speculative and requires further investigation.

6.4 CONCLUSIONS

The main conclusions from this chapter are as follows:

1. Preservative-induced adaptive resistance involved more significant gene expression changes than those provoked by exposure to sub-inhibitory concentrations of DMDM hydantoin and the isothiazolone blend MIT/CMIT.

2. The exposure of *B. lata* strain 383 to sub-MIC of DMDM hydantoin and MIT/CMIT did not elicit large changes in gene expression, or induce the differential expression of key putative resistance determinants that were identified in the transposon mutagenesis study (Chapter 5). This may have been a result of the test preservative concentrations being too low, or of the combined action of several small changes in the gene expression contributing to the resistance of *B. lata* strain 383 to low levels of the preservatives.

3. Gene expression changes in the isothiazolone-adapted *B. lata* strain 383 were stable irrespective of the presence of the priming preservative.

4. Transcriptomic analysis revealed that isothiazolone-induced adaptive resistance in *B. lata* strain 383 is multi-factorial in nature.

5. A novel putative role for an RND-efflux system (B1004_B1006 genes) was identified in relation to isothiazolone-induced adaptive resistance in *B. lata* strain 383.

6. A novel putative role for an ABC-type transporter system, homologous to the *P. aeruginosa* Ttg2 system involved in resistance to organic solvents, was identified in relation to isothiazolone-induced adaptive resistance to in *B. lata* strain 383.

7. The susceptibility of the preservative-adapted *B. lata* strain 383-CMIT to the isothiazolone preservative blend and ciprofloxacin antibiotics, increased, but failed to revert to wild-type levels in the presence of an efflux inhibitor, thereby suggesting the involvement of other resistance determinants.

8. The increased expression of superoxide dismutase and paraquat-inducible protein genes in the preservative-adapted derivative, suggests that prolonged exposure to MIT/CMIT may generate ROS, and that the induction of defence mechanisms against oxidative stress may contribute to the adaptive-resistance of *B. lata* strain 383.

9. A significant decrease in the expression of genes putatively associated with the biosynthesis and transport of the siderophore pyochelin may suggest a depletion of assimilable sulphur in the isothiazolone-adaptive *B. lata* strain 383-CMIT derivative.

7 GENERAL CONCLUSIONS AND FUTURE RESEARCH

7.1 CONCLUSIONS

A novel and systematic study of the preservative susceptibility of *Burkholderia cepacia* complex bacteria was undertaken. The activity of several preservatives on a genetically diverse panel of Bcc strains, which took into consideration recent changes in taxonomy, was evaluated, and the role of Bcc species diversity in preservative susceptibility was explored. A genome sequenced *B. lata* strain that represented a Bcc species commonly encountered in the environmental-industrial niche was selected as a suitable model strain. Transposon mutagenesis and transcriptomic analysis of global gene expression was successfully used to investigate the molecular basis for preservative resistance and preservative-induced adaptive resistance.

In this chapter, the general conclusions of each main body of work will be reiterated with respect to the aims and hypotheses stated in Chapter 1. In addition, the main areas of future research generated from these findings will be discussed.

1) **<u>Hypothesis 1:</u> the preservative susceptibility of Bcc bacteria is related to species diversity and source of isolation**.

Despite Bcc bacteria being recognised as a predominant bacterial contaminant of preserved raw materials and finished products in industry, Bcc species diversity within the environmental-industrial niche is poorly understood. Bcc bacteria encountered as contaminants are not routinely identified to the species or strain level and the publication of instances of Bcc contamination are not a priority for manufacturers. The relationship between preservative susceptibility and Bcc taxonomy had not been extensively studied.

The present study utilised MLST data analysis to expand current knowledge of Bcc species diversity in the environmental-industrial niche, and identified *B. lata* and *B. cenocepacia* as commonly encountered species groups. This indicated that the currently recommended *B. cepacia* challenge test organisms may not represent the diversity of Bcc species that are commonly encountered as industrial contaminants. In light of this, a collection of Bcc strains

that represented the species diversity encountered in the environmental-industrial niches were recommended as potential future challenge test organisms (Table 15).

The relationship between preservative susceptibility, Bcc species diversity and source of isolation was systematically investigated using a collection of genetically diverse Bcc strains that accounted for recent changes in taxonomy and various isolation sources. Preservative susceptibility was observed not to be related to species diversity, as susceptibility varied both between and within species groups. In addition, Bcc preservative susceptibility was observed not to be related to the source of isolation for seven of the eight preservatives evaluated. However, Bcc isolates from the environmental-industrial niche were observed to be significantly less susceptible to DMDM hydantoin than Bcc from clinical and environmental isolation sources. This suggested that the use of DMDM hydantoin in the environmental-industrial niche may select for Bcc that are highly tolerant of this preservative group.

Overall, the limited survey of industrial preservative resistance performed herein, indicated that it was not linked to specific Bcc species, therefore the stated hypothesis is rejected.

2) **<u>Hypothesis 2:</u> the stepwise exposure of *B. lata* strain 383 to sub-lethal preservative concentrations will promote stable adaptive resistance; in addition, this preservative-induced adaptive resistance will confer cross-resistance to other antimicrobials.**

Preservatives are often used at concentrations far lower than that of disinfectants or antiseptics. As a result, the risk of exposure to sub-inhibitory concentrations of preservatives may be considered greater than that of disinfectants. Although it is stringently avoided, the exposure of contaminating bacteria to sub-inhibitory concentrations may occur as a result of the inadequate sanitation of production lines, the degradation of preservatives by other microbial contaminants, or by the detrimental activity of formulation excipients (Orth *et al.*, 2006). Bacterial adaptation to sub-inhibitory concentrations of antimicrobials is well documented but the competency of Bcc to adapt to preservatives, and the mechanisms leading to Bcc adaptive-resistance, had not been systematically studied.

The present study investigated preservative-induced adaptive resistance of Bcc bacteria via the progressive subculture of *B. lata* strain 383, in sub-inhibitory preservative concentrations.

Stable adaptive-resistance to isothiazolone and benzethonium chloride preservatives was induced in *B. lata* strain 383 by this method. The antimicrobial susceptibility profile, and level of susceptibility, of the four preservative-adapted derivatives varied, this suggested that the mechanisms leading to preservative-induced adaptive resistance in *B. lata* strain 383 may be agent specific. Stepwise exposure to sub-inhibitory preservative concentrations did not elevate resistance above that of the maximum preservative concentrations regulated for use in the personal care industry. However, the induction of stable adaptive-resistance mechanisms via exposure to sub-inhibitory concentrations may play an important role in the selection and development of preservative resistant Bcc in the environmental-industrial niche. As any stable reduction in susceptibility can translate into biological significance by facilitating the acquisition of additional resistance traits that may lead to the development of high-level resistance. Knowledge that may be of significant benefit to manufacturers is that phenoxyethanol, methyl paraben and DMDM hydantoin were identified as putatively recalcitrant to *B. lata* strain 383 adaptation. However, the high level of native resistance to DMDM hydantoin observed in Bcc isolates from the environmental-industrial niche also indicates that the agent is not recalcitrant to adaptation in real situations.

Cross-resistance to other antimicrobials, including antibiotics, is a phenomenon commonly encountered in experiments of biocide-induced adaptive resistance. This is of particular concern as biocides are extensively, and often indiscriminately, used in domiciliary, clinical and industrial settings. The present study demonstrated that isothiazolone-induced adaptive resistance conferred stable cross-resistance to other preservatives of the same class, suggesting that preservative-induced adaptation to one agent may diminish the efficacy of others. In addition, two of the isothiazolone-adapted *B. lata* strain 383 derivatives demonstrated stable cross-resistance to fluoroquinolone antibiotics; which was mediated via a non-specific resistance mechanism not a specific modification of the antibiotic target. However, elevated levels of resistance to fluoroquinolone antibiotics were accompanied by an increase in susceptibility to the aminoglycoside antibiotic amikacin. Overall, preservative-induced adaptive-resistance in *B. lata* strain 383 was not associated with multi-drug resistance indicating that greater overall fitness was not promoted by preservative exposure.

Stable adaptive resistance to preservatives was promoted in *B. lata* strain 383. This conferred stable cross-resistance to other agents, but not overall increased multi-drug resistance; therefore the stated hypothesis is accepted.

3) <u>Hypothesis 3</u>: Bcc resistance to isothiazolinone and DMDM hydantoin preservatives is mediated by multiple resistance determinants.

Bcc bacteria are renowned for high levels of intrinsic resistance to antimicrobials. Although there have been great advances in the characterisation of antibiotic resistance mechanisms, the molecular basis for preservative resistance had not been extensively studied. In contrast to antibiotics, the antimicrobial action of the majority of preservatives is considered to be multi-factorial in nature. Understanding the interaction between Bcc bacteria and preservatives, and the identification of the resistance determinants, is essential in order to better target these organisms and to facilitate the implementation of improved preservative strategies which target resistance mechanisms.

In order to discover genes and gene pathways that encode Bcc resistance determinants for isothiazolone and DMDM hydantoin preservatives, *B. lata* strain 383 was subjected to transposon mutagenesis using the plasposon pTn*Mod*OTp'. The identified preservative resistance determinants of *B. lata* strain 383 were multi-factorial in nature, and a single target for preservatives was not apparent. Resistance to the released formaldehyde from DMDM hydantoin may involve its detoxification by a glutathione-dependent formaldehyde dehydrogenase pathway. A novel putative role of a type II secretion system was identified in relation to DMDM hydantoin and methylisothiazolinone resistance. While several efflux transporter systems, belonging to several superfamilies, were identified as putative resistance determinants in relation to the isothiazolone blend. This included a novel putative role for a homologue of an ABC-type transport system being associated with resistance to organic solvents. Bacterial defence mechanisms against oxidative stress were also identified in relation to isothiazolone preservatives.

Several putative resistance determinants were identified in relation to DMDM hydantoin and isothiazolone preservatives; therefore, the stated hypothesis is accepted.

4) **<u>Hypothesis 4:</u> multiple preservative resistance determinants will be identified in *B. lata* strain 383 by the differential expression of genes and gene pathways in response to sub-inhibitory concentrations of preservative.**

Investigating how Bcc bacteria deal with the stresses associated with exposure to preservatives is fundamental to understanding why Bcc can successfully contaminate and grow in preserved raw materials and finished product in industry. The present study used the genome sequence *B. lata* strain 383 as a model strain for this area of research. Given that *B. lata* strain 383 has a large genome of 8.67 Mb (Mahenthiralingam, 2007) and the potential to possess multiple genetic pathways for a given function, transcriptomics profiling was selected as a suitable high-throughput approach to investigate the response of *B. lata* strain 383 to sub-MIC of DMDM hydantoin and isothiazolone MIT/CMIT preservatives.

The analysis of global gene expression revealed few significant changes in response to sub-MICs of both preservatives. This suggests that either the combined contributions of minor changes in the expression of *B. lata* strain 383 resistance determinants was sufficient to resist sub-MIC levels, or that the evaluated concentrations were insufficient to induce large transcriptional alterations. The expression of key putative resistance determinants that were identified in the transposon mutagenesis study did not significantly alter in response to sub-MICs of both preservatives. This suggests that either the putative key resistance determinants identified were not primary defence mechanisms in *B. lata* strain 383, or that their differential expression was not induced at the low concentrations that were evaluated.

Transcriptomic profiling identified multiple putative resistance determinants of *B. lata* strain 383; therefore, the stated hypothesis is accepted.

5) **<u>Hypothesis 5:</u> multiple resistance determinants leading to preservative-induced adaptive resistance in *B. lata* strain 383 will be identified by the relative differential expression of genes and gene pathways in a preservative-adapted derivative of the wild-type.**

The molecular mechanisms leading to bacterial adaptation to antimicrobials have only recently started to be understood. Although not yet fully characterised in many bacteria, it is apparent that adaptive-resistance is more complex than initially thought, often involving intricate regulatory responses (Fernández *et al.*, 2011). Global gene modulation and the resistance mechanisms involved in preservative-induced adaptive resistance in Bcc bacteria had not been extensively studied.

A transcriptomic microarray-based approach was successfully used to gain insight into global changes in gene expression associated with isothiazolone-induced adaptive resistance in *B. lata* strain 383. Changes to the transcriptome were stably maintained in the absence of the priming preservative, and several resistance determinants that were multi-factorial in nature were identified by transcriptomic analysis. A novel putative role for a RND-efflux system (B1004_B1006 genes) was identified as an adaptive-resistance mechanism, whose expression was not observed to alter in the parental strain on a single sub-MIC exposure. Additional gene expression analysis suggested the over-expression of the homologue RND-efflux component gene in *B. lata* strains from the environmental-industrial niche with native elevated levels of isothiazolone resistance. Global gene expression analysis of the preservative-adapted *B. lata* strain 383 derivative also revealed the increased expression of a second active efflux system belonging to the ABC-type efflux family that had been identified as a putative resistance determinant by transposon mutagenesis (A3512_A3517 genes). This suggested that active efflux is putatively a key isothiazolone-induced adaptive resistance mechanism of *B. lata* strain 383, and therefore a potential target to improve the efficacy of isothiazolone preservatives against Bcc bacteria. The susceptibility of the preservative-adapted derivative was observed to increase in the presence of an efflux inhibitor, but failed to revert to wild-type levels, therefore suggesting the involvement of additional determinants in adaptive-resistance.

Transcriptomic profiling identified multiple putative resistance determinants of the preservative-adapted *B. lata* strain 383-CMIT derivative; therefore, the stated hypothesis is accepted.

7.2 FUTURE RESEARCH

The following section discusses the main areas of future research that were generated from the findings of the present study. Other areas worthy of further investigation are discussed in previous chapters.

1. Further genetic typing studies are required to explore differences in the distribution of Bcc species in the environmental-industrial niche at various geographical locations, and to identify prevalent sequence types in industry. The identification of prevalent or globally distributed sequence types may impact on current challenge tests, as their inclusion as 'in-house' native adapted Bcc test organisms may be beneficial to manufacturers. The identification of geographical differences in Bcc species distribution may also be beneficial, enabling manufacturers to challenge preservative systems with test organisms representative of prevalent regional Bcc species or sequence types.

2. Current preservative efficacy testing procedures usually involve the traditional, and time consuming plate count method. Rapid procedures that exploit established technologies such as ATP-based bioluminescence, impedance-based testing, and flow cytometery-based assays provide economic benefit by reducing time-of-testing but have inherent disadvantages such as, requiring a pre-enrichment step and the lysis of cells, being non-discriminative in that growth of any microorganism will trigger a positive response, or a reliance on a stain or dye as the signal device (Orth *et al.* 2006). The application of bacterial bioluminescence in preservative efficacy testing procedures may provide a rapid means to quantify viable CFU directly via light emission without additional pre-enrichment, cell labelling or the need for exogenous substrate. The use of bioluminescence bacteria, engineered to express *lux* genes, as reporters of various microbial phenomenon is well documented (Van Der Meer & Belkin, 2010), and has been shown to be a sensitive and real-time reporter of antimicrobial efficacy (Dhir & Dodd, 1995; Marques *et al.*, 2005; Thorn *et al.*, 2007) with excellent correlation between bioluminescence and traditional plate count data. Light emission from self-bioluminescent bacteria is directly linked to metabolism and can be accurately quantified with a high level of sensitivity, in a non-destructive manner and in real-time, using a luminometer. Recombinant reporter Bcc strains, and

other relevant bacterial species, that constitutively express the *Photorhabdus luminescence lux* operon (Meighen, 1991) could be constructed and utilised in future challenge tests.

3. The preservative-adapted derivatives of *B. lata* strain 383 demonstrated stable alterations in susceptibility and several phenotypic traits. A transcriptomic microarray-based approach was successfully used to investigate changes in the global gene expression of the *B. lata* strain 383-CMIT derivative, and identified putative resistance determinants via stable alterations to the transcriptome. Genome re-sequencing of the adapted derivatives would add to this dataset and may reveal any mutations, and/or genomic rearrangements that occurred as a result of prolonged exposure to sub-MIC of preservatives.

4. Several key genetic pathways were identified in the present study as putative preservative resistance determinants of *B. lata* strain 383, via transposon mutagenesis and transcriptomic analysis. In particular, the role of the RND-efflux system (B1004_B1006 genes), the ABC-efflux system (A3512_A3517 genes) and the type II secretion system (A3244_A3233 genes) in preservative resistance, requires confirmation via the generation of non-polar/site-directed gene mutations and complementation studies.

5. Active efflux was identified as a putative key isothiazolone resistance mechanism in *B. lata* strain 383, and consequently efflux-system pose a potential target to improve the efficacy of isothiazolone preservatives against Bcc bacteria. The present study demonstrated an encouraging increase in the susceptibility of a preservative-adapted *B. lata* strain 383 derivative in the presence of the efflux inhibitor phenylalanine arginine β-naphthylamide (MC-207,110). This warrants the evaluation of other compounds designed and synthesised to inhibit bacterial efflux pumps (Pagès *et al.*, 2005), and those derived from natural sources (Stavri *et al.*, 2007).

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9 APPENDICES

9.1 APPENDIX 1: THE SEQUENCE ALIGNMENT OF THE QRDR OF *B. LATA* STRAIN 383 AND PRESERVATIVE-ADAPTED DERIVATIVES

The aligned consensus sequences of the QRDR of the topoisomerase genes in *B. lata* strain 383 and its preservative-adapted derivatives, are provided on the CD-ROM.

9.2 APPENDIX 2: GENE LISTS DERIVED FROM *B. LATA* STRAIN 383 MICROARRAY DATA WITH THE APPLICATION OF A 1.5-FOLD CHANGE CRITERION

Gene lists generated from *B. lata* strain 383 microarray data with the application of a 1.5-fold change, are provided on the CD-ROM. Raw microarray data are available at Array express (http://ww.ebi.ac.uk/arrayexpress/) under the accession number E-MEXP-2827.