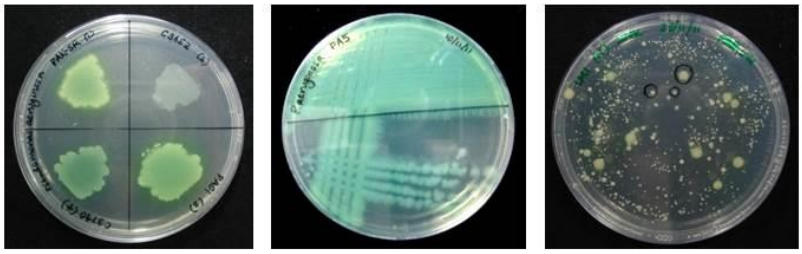
The resistance of *Pseudomonas aeruginosa* to preservatives used in industrial formulations



Thesis presented for the Degree of Philosophiae Doctor
by

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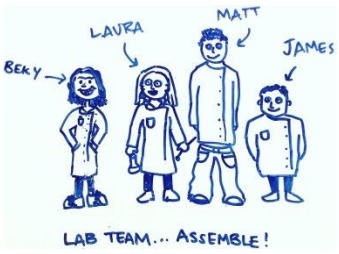

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Scientific Conferences and Awards

Methods for the detection of *P. aeruginosa* (2012). MITReG Postgraduate Research Day, Cardiff. Poster presentation. Awarded joint 2nd prize for poster presentations.

Evaluation of selective media and genetic markers for the detection of *P. aeruginosa* (2013). FEMS Microbiology Congress 2012, Leipzig, Germany. Poster presentation. Awarded the Welsh Livery Guild Travel Scholarship.

Detection & diversity of *P. aeruginosa* in industrial settings (2013). 14th International conference on *Pseudomonas*, Lausanne, Switzerland. Poster presentation.

Detection & diversity of *P. aeruginosa* in industrial settings (2013). MITReG Research Symposium, Swansea. Oral presentation. Awarded 1st prize winner for oral presentations.

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Detection & diversity of *P. aeruginosa* in industrial settings (2014). ASM conference, Boston, Massachusetts. Poster presentation.

Publications

Weiser, R., Donoghue, D., Weightman, A. and Mahenthiralingam, E. (2014). Evaluation of five selective media for the detection of *Pseudomonas aeruginosa* using a strain panel from clinical, environmental and industrial sources. *Journal of Microbiological Methods* **99**:8-14.

Cullen, L., Weiser, R., Olszak, T., Maldonado, R., Moreira, A. S. *et al.* (2015). Phenotypic characterisation of an international *P. aeruginosa* reference panel: Strains of cystic fibrosis origin show less *in vivo* virulence than non-CF strains. *Microbiology* **161**(10):1961-1977

Summary

P. aeruginosa is a versatile microorganism with high levels of antimicrobial resistance and a common contaminant of home and personal care (HPC) products. Relatively little is known about *P. aeruginosa* in industrial settings and to further investigate this, four areas were considered: (i) culture-dependent and –independent detection of *P. aeruginosa*; (ii) *P. aeruginosa* and bacterial diversity in HPC products; (iii) preservative susceptibility and phenotypic characterisation of industrial *P. aeruginosa* strains, and; (iv) *P. aeruginosa* adaptation to a preservative combination.

A large collection of *P. aeruginosa* and non-*P. aeruginosa* industrial isolates was utilised to evaluate five selective agars for the detection of *P. aeruginosa.* Media using negative selection performed best overall, but media using positive selection had potential as enrichment media. Culture-independent detection of *P. aeruginosa* and overall bacterial diversity was achieved via direct DNA extraction from contaminated HPC products, species specific PCRs and 16S rRNA gene sequencing analysis. The bacterial diversity in HPC products was low, with less than three contaminating genera in each product. The diversity of *P. aeruginosa* strains from industrial, clinical and environmental sources was investigated using five genotyping techniques, ranging from PCR-fingerprinting methods to whole genome sequencing, and phenotypic assays examining preservative susceptibility, growth dynamics and motility. *P. aeruginosa* strain diversity was high and there was no association between genotype, phenotype and isolation source. The development of adaptive resistance of *P. aeruginosa* to a preservative combination used in HPC products was modelled using planktonic growth and biofilm assays. *P. aeruginosa* PA14 grew in elevated levels of the preservatives chloromethylisothiazolinone, methylisothiazolinone and dimethylol dimethyl hydantoin but it was unknown whether the increased tolerance was stable.

Whilst eradication of *P. aeruginosa* from the industrial environment is unlikely, improved detection methods and understanding of the species will inform industrial practices and preservative formulations to minimise HPC product contamination.

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Abbreviations

AMEs	Aminoglycoside-modifying enzymes
ATCC	American Type Culture Collection
AT typing	Clondiag Array Tube typing
ATP	Adenosine triphosphate
A_w	Water activity
BA	Benzoic acid
BCC	Cardiff University strain collection
BIGSdb	Bacterial Isolate Genome Sequence Database
BIT	Benzisothiazolinone
BLAST	Basic Local Alignment Search Tool algorithm
BSM-G	Basal salts medium supplied with 0.4% (w/v) glucose
CF	Cystic fibrosis
Cfu/g	Colony forming units per gram
Cfu/ml	Colony forming units per millilitre
Chrom ID Pa	chromID® *P. aeruginosa*
CHX	Chlorhexidine
CITMIT	Chloromethylisothiazolinone
CITMIT-DMDMH-EDTA	Preservative combination with 0.0009% CITMIT, 0.22% DMDMH and 0.1% EDTA
CLIN	Clinical
COPD	Chronic obstructive pulmonary disorder
DMDMH	Dimethyloldimethyl hydantoin
DMSO	Dimethylsulphoxide
DNA	Dioxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DWL	Dish wash liquid
eBURST	Enhanced version of the based upon related sequence types algorithm
EC	European Commission
EDTA	Ethylenediamine tetracetic acid
ENA	European nucleotide archive
ENV	Environmental
EP	European Pharmacopeia
ESBLs	Extended spectrum β-lactamases
FDA	U.S. Food and Drug Administration
FIC	Fractional inhibitory concentration
FWS	Fabric wash
wgMLST	Whole genome multilocus sequence typing
GMP	Good manufacturing practices
HPC	Home and personal care

IMG	Integrated microbial genomes
ID	Identification
IND	Industrial
JP	Japanese Pharmacopeia
Kb	Kilobase pairs
KER	Keratitis
LAC	Liquid abrasive cleaner
LB	LB broth
LMG	Belgian co-ordinated collections of micro-organisms, Gent
MBLs	Metallo β-lactamases
MH broth	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MIT	Methylisothiazolinone
MLST	Multilocus sequence typing
MWF	Metal working fluid
mZ-agar	Modified Z-agar
NCTC	National Collection of Culture Types
NPV	Negative predictive value
OD	Optical density
OTU	Operational taxonomic unit
PaβN	Phenyl-arginine-beta-napthylamide
PCN	Pseudomonas CN selective agar
PCPC	Personal Care Products Council
PCR	Polymerase chain reaction
PC	Personal care
PET	Preservative efficacy testing
PFGE	Pulsed field gel electrophoresis
PHE	Phenoxyethanol
PIA	Pseudomonas isolation agar
PPV	Positive predictive value
QAC	Quaternary ammonium compound
qPCR	Quantitative PCR
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA (typing)
RAPEX	Rapid Alert System for non-food consumer products in the EU
RDP	Ribsomal Database Project
RGP	Regions of genomic plasticity
RISA	Ribosomal intergenic spacer analysis
rMLST	Ribosomal multilocus sequence typing
RNA	Ribonucleic acid
RND	Resistance nodulation division

rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SC	Surface cleaner
ST	Sequence type
Taq	*Thermus aquaticus*
TC	Timber care
TSA	Tryptone soya agar
TSB	Tryptone soya broth
SCCS	Scientific Committee on Consumer Safety
USP	United States Pharmacopeia
UV	Ultra violet
URDPS	Unilever Research and Development Port Sunlight
VBNC	Viable but not cultivable
VSC	Velsan SC preservative enhancer
WC	Wood cleaner
WGS	Whole genome sequencing

1. Introduction

1.1 Microbial contamination in the home and personal care (HPC) industry

1.1.1 Microbial contamination of HPC products

The HPC industry manufactures a diverse range of products which are sold globally and used daily by consumers. Personal care products, such as cosmetics and toiletries are used to beautify or maintain personal hygiene (Perry 2001), whilst household care products are generally used to clean or freshen home surfaces and clothing. A large variety of product formulations are encountered within the HPC industry and are tailored to suit the application. Factors such as water activity (A_w; the water content available to microbial utility) (Lundov *et al.* 2009), pH, redox potential and nutrient availability all influence a product's propensity to support microbial growth (Orth *et al.* 2006). HPC products with a water activity above 0.60 and a pH between 4.0 and 10.0 are particularly at risk of contamination (Orth *et al.* 2006).

Although HPC products are not expected to be sterile, they are required to be 'safe for their intended purpose under normal and foreseeable conditions of use' (Orus and Leranoz 2005). Microbial contamination can have adverse effects which are damaging to the product, the manufacturer and the consumer. Product spoilage by the presence of and/or growth of microorganisms renders HPC products unfit for use (Smart and Spooner 1972). A range of undesirable effects can be observed including changes in odour, colour, viscosity and the production/utilisation of gas leading to the warping of packaging. In addition, product performance may be affected such as a decrease in the effectiveness of active ingredients, a loss of lathering activity (Perry 2001) or the breakdown of emulsions (Smart and Spooner 1972). Economic losses to the manufacturer can result through loss of contaminated raw materials or batches of finished product, and the investigation and decontamination of production processes. Furthermore, contaminated products and product recalls can affect product marketability and consumer satisfaction (Orth *et al.* 2006) which impact the reputation of a company or brand name.

More seriously, contamination represents a health risk to vulnerable consumers, and the presence of opportunistic pathogens in HPC products has been linked with infections in nosocomial settings. These incidences include *Pseudomonas aeruginosa* contamination of hand lotion and mouthwash (Stephenson *et al.* 1984; Becks and Lorenzoni 1995), *Burkholderia* in

mouthwashes (Matrician *et al.* 2000; Molina-Cabrillana *et al.* 2006; Kutty *et al.* 2007) and moisturising lotion (Álvarez-Lerma *et al.* 2008), and *Serratia marcescens* in soap and detergents (Polilli *et al.* 2011). Individual infections contracted from contaminated products outside of the hospital environment are less likely to be documented (Lundov, 2009) although contaminated products do reach the market place, as product recall data (Jimenez 2007; Lundov and Zachariae 2008; Sutton and Jimenez 2012) and surveys of off-the-shelf products have shown (Lundov *et al.* 2009).

Contamination of HPC products may arise via two routes, through manufacture and subsequent storage, or through consumer use (Farrington *et al.* 1994). To minimise contamination during manufacture, Good Manufacturing Practices (GMP) are followed by industries, stipulated by regulatory bodies such as the U.S. Food and Drug Administration (FDA) and within the European Commission (EC) Cosmetics Directive. These guidelines cover all aspects of manufacture including quality control and handling of raw materials, manufacturing facilities and equipment, cleaning and sanitisation procedures, and personnel training. Whilst products may never be completely sterile, GMPs help to ensure benign levels of intrinsic contamination and the absence of harmful microorganisms (Perry 2001). Consumer use leads to contamination through repeated inoculation of the product with the skin microbiota/body fluids or with microorganisms from the domestic environment (Perry 2001; Orth *et al.* 2006). This unavoidable occurrence is counteracted by careful product formulation and the inclusion of preservatives to prevent microbial proliferation (Orth *et al.* 2006). In addition, to further protect the consumer, the EU has introduced 'best used before' and 'period after opening' guidelines which are provided on packaging to advise on product lifespan (Orus and Leranoz 2005; Lundov *et al.* 2009).

1.1.2 Microbiological quality assurance and detection of HPC product contamination

To ensure the safety and stability of HPC products, microbiological limit testing is performed by manufacturers to ensure that total microbial counts are low and products are devoid of objectionable species. As there are no formal regulations for microbial limits, companies generally follow recommendations by professional associations such as the FDA, the Personal Care Products Council (PCPC), the Scientific Committee on Consumer Safety (SCCS) and Cosmetics Europe (Table 1.1), and may even enforce stricter in-house regulations than suggested. Detection is predominantly culture-dependent with total viable counts of aerobic mesophilic microorganisms (bacteria, yeast and mold) being achieved by traditional plate counts, and identification of objectionable species based on phenotypic and biochemical testing

(Orus and Leranoz 2005; Orth *et al.* 2006). The term 'objectionable organism' is slightly ambiguous (Sutton and Jimenez 2012), and according to the PCPC definition means 'an organism that can be harmful to the user based upon the nature of the product, its intended use and its potential hazard, or is able to compromise the physical integrity or appearance of the product'. The species in Table 1.1 are specified objectionable organisms, but are certainly not the only problematic contaminants (Sutton and Jimenez 2012). This poses an issue for manufacturers who must consider which other species should be excluded from products. Suggestions for two further specified objectionable bacteria include *Burkholderia spp.* and *Bacillus cereus* (Sutton 2012).

To determine whether a product formulation is adequately preserved to prevent microbial contamination, preservative efficacy testing (PET), also known as 'challenge testing', is performed. Evaluation of the complete formulation is required, as the concentrations of preservatives sufficient for growth inhibition in the laboratory may not reflect their performance in the product (Orth *et al.* 2006). The general PET method involves the inoculation of product samples with microorganisms, followed by the removal of aliquots at stipulated time-points and the enumeration of survivors, in order to determine the overall reduction in viable cell numbers (Russell 2003). Several standardised methods of PET exist, for example those provided by the United States Pharmacopeia (USP), the European Pharmacopeia (EP) and the Japanese Pharmacopeia (JP). In addition, in-house methods adapted from the Pharmacopeia methods are sometimes used (Orth *et al.* 2006). Variables within the methods include choice of microorganism, the number and frequency of sampling points after inoculation, and the limits determining adequate preservation (Orth *et al.* 2006). The microorganisms used in PET are those likely to contaminate products during use, such as *S. aureus*, *P. aeruginosa*, *E. coli*, *Burkholderia spp.*, Aspergillus *niger* and *C. albicans* (Russell 2003). Both organisms from culture collections and 'in-house' isolates which may have been isolated from contaminated products or the production plant are usually used (Orus and Leranoz 2005).

As stated above a cultivation-based approach is routinely used in industry to detect microbial contaminants (Jimenez 2001a), which appears to generally provide satisfactory quality control for HPC products (Orth *et al.* 2006). There are a number of drawbacks to culture-dependent methods, however, including lengthy processing times, the expense of media and reagents, and the difficulties associated with the detection of fastidious, 'stressed' or viable but not cultivable (VBNC) cells (Orus and Leranoz 2005). Alternative 'rapid' detection methods such as ATP-bioluminescence, impedance and flow cytometry, rely on the metabolic state of microorganisms, can take less than 24 hours and are more reliable for detecting stressed cells (Jimenez 2001b;

Orus and Leranoz 2005). Whilst these methods confer economic benefits associated with a reduction in testing times, they only estimate the total bioburden, and further tests would still be required to identify specific groups of microorganisms (Orus & Leranoz, 2005). In addition, the ATP-bioluminescence method can result in false positive results through the detection of non-microbial ATP in the detection system, or false negatives through product ingredients which can limit, or 'quench', the reaction (Orth *et al.* 2006). Other molecular methods such as species-specific polymerase chain reaction (PCR)-based assays and immunoassays have been suggested for species identification in this context (Jimenez 2001b).

Table 1.1 Microbial limits and specified objectionable microorganisms for personal care products

Organism	**Product category**	**Professional association recommendations**			
		PCPC (USA)[1]	FDA (USA)[2]	SCCS (EU)[3]	Cosmetics[4] Europe (EU)
Total aerobic mesophilic counts	Product for children under 3 years	< 100-500 cfu/ml or cfu/g	-	100 cfu/ml or cfu/g	< 100-500 cfu/ml or cfu/g
	Product for use in the eye area/on mucous membranes	< 100-500 cfu/ml or cfu/g	500 cfu/ml or cfu/g	100 cfu/ml or cfu/g	< 100-500 cfu/ml or cfu/g
	All other products	< 1000-5000 cfu/ml or cfu/g	1000 cfu/ml or cfu/g	1000 cfu/ml or cfu/g	< 1000-5000 cfu/ml or cfu/g
Specified microorganisms	Product for children under 3 years	Absence of *P. aeruginosa*, *S. aureus* and *E. coli*	Absence of *P. aeruginosa*, other *Pseudomonas spp.*, *S. aureus*, *Streptococcus pyogenes* and *Klebsiella pneumoniae*	Absence of *P. aeruginosa, S. aureus* and *C. albicans* in 1 ml or 1 g	Absence of *P. aeruginosa, S. aureus* and *C. albicans* in 0.1 ml or 0.1 g
	Product for use in the eye area/on mucous membranes				
	All other products			Absence of *P. aeruginosa, S. aureus* and *C. albicans* in 0.1 ml or 0.1 g	

Footnotes:
[1]Personal Care Products Council (PCPC; formerly CFTA), Microbiology Guidelines 2007, Section 12 Establishing Microbial Quality of Product
[2] U.S. Food and Drug Administration (FDA), Bacteriological Analytical Manual Chapter 23 Microbiological Methods for Cosmetics, C. Interpretation
[3]Scientific Committee on Consumer Safety (SCCS) Notes of guidance for testing of cosmetic substances and their safety evaluation 8th Revision
[4]Cosmetics Europe (formerly COLIPA), Guidelines on Microbial Quality Management, (MQM) Edition of 1997

1.1.3 Commonly encountered contaminants in the HPC industry

As there is no requirement for industry to publish information concerning contamination incidents, systematic literature in this area is limited. Product recall data, however, is collated by the FDA and the European Commission and made publicly available. Reports based on FDA data for personal care and non-sterile pharmaceutical products (Jimenez 2007; Sutton and Jimenez 2012) and the Rapid Alert System for non-food consumer products in the EU (RAPEX) (Wong *et al.* 2000; Lundov *et al.* 2009) indicate the range of microorganisms found as contaminants. Figure 1.1 illustrates the different types and proportions of microorganisms associated with non-sterile products and was compiled with data from two published studies which surveyed FDA product recalls (Jimenez 2007; Sutton and Jimenez 2012), and data directly obtained from the EU RAPEX website (www.ec.europa.eu/consumers/consumers_safety/safety_products/rapex/index_en.htm) from 2005-2014 having a microbiological risk type.

The most common causes of product recalls were unacceptably high levels of unidentified aerobic mesophilic microorganisms, fungal (yeast or mold) growth, and the bacterial contaminants *P. aeruginosa* and *Burkholderia* spp. (see Figure 1.1). Although the FDA and RAPEX reports differ in the relative proportions of groups of contaminating microorganisms, Gram-negative bacterial species are particularly prevalent in industry. One report (Jimenez 2007) documented that Gram-negative bacteria accounted for 60% of product recalls, with fungal and Gram-positive bacterial species accounting for approximately 25% and 5%, respectively. It is speculated that this bias could be due to the association of Gram-negative bacterial species with water contamination, as water is one of the most common raw materials used in HPC manufacture (Jimenez 2007). Whilst diverse microorganisms can contaminate HPC products, the majority of incidents are linked to only one species. Reports based on FDA data has shown co-contamination represents about 5% of the total recalls (Jimenez 2007; Sutton and Jimenez 2012), and EU RAPEX data suggests slightly higher at 10-15%. In the majority of co-contamination incidents only 2 species were reported as present, and the highest number of different species isolated from a single product was 5 (Jimenez 2007).

It is not always easy to interpret the product recall data, as there is no indication of how thorough the identification processes have been, or whether incidents citing 'aerobic mesophilic microorganisms' as the reason for recall involve multiple species. In addition, using only culture-dependent techniques for identification may exclude fastidious or uncultivable isolates (Orus and Leranoz 2005). We also have very little knowledge about industrial microorganisms below the species level, for example whether certain strains are more prevalent in industry than

others. With the exception of an incident where a contaminated mouthwash led to a multi-state outbreak of *Burkholderia* infection in the USA (Kutty *et al.* 2007), few studies have sought to apply epidemiological techniques to link strain types to contaminated products and/or potential sources of contamination. Dedicated studies to assess the diversity of microbial contamination in industry would therefore be incredibly informative.

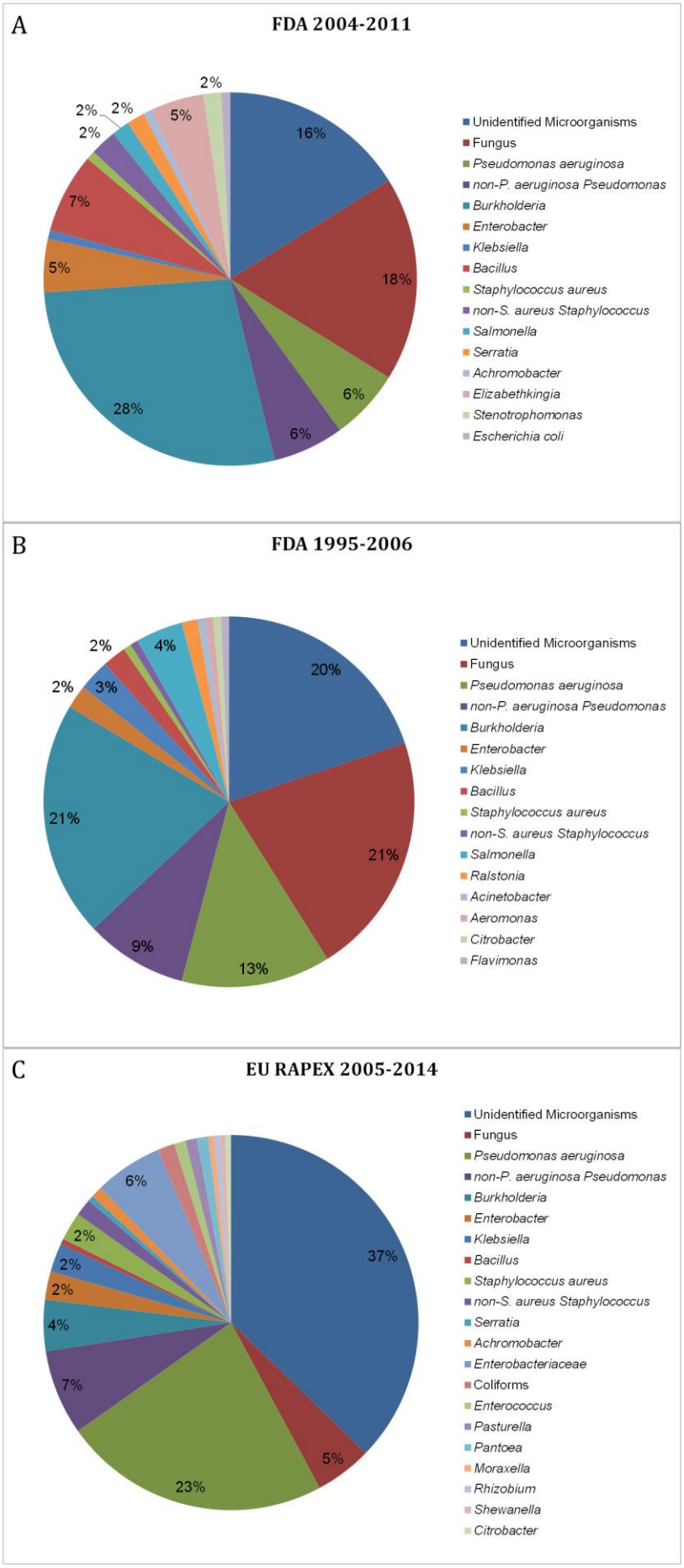


Figure 1.1 Microorganisms associated with non-sterile product recalls. The different types of micro-organisms, and the number of times each group was linked to a contamination incidents is depicted graphically for (A) FDA data from 2004-2011 for 143 product recalls (Sutton and Jimenez 2012), (B) FDA data from 1995-2006 for 134 product recalls (Jimenez 2007), (B) FDA data from 1995-2006 for 134 product recalls, and (C) EU RAPEX data from 2005-2014 for 176 product recalls with a microbiological risk type (collated in this study). Percentage values are given for groups whose prevalence exceeded 1%.

1.1.4 Preservation of HPC products

Preservatives belong to a class of chemical agents known as biocides which exhibit broad spectrum antimicrobial activity (McDonnell and Russell 1999). Other categories of biocides include antiseptics, which are used on living tissues, and disinfectants, which are used on inanimate objects (Orth *et al.* 2006). Whilst certain chemical compounds can be used for all three applications, those used for preservation are generally incorporated into pharmaceutical, cosmetic or other product types at low levels to prevent microbial spoilage (Orth *et al.* 2006). Preservatives are not added to products to compensate for inadequate manufacturing conditions, rather to prevent the proliferation of contaminants introduced by the consumer after manufacture (Hiom 2008). There are high expectations of preservatives: to demonstrate broad spectrum antimicrobial activity at permitted levels; to maintain activity throughout the lifespan of the product; to not compromise product quality or performance, and; to not pose a risk to consumer health (Elder and Crowley 2012). Choosing preservatives which encompass all of these attributes is difficult as a number of factors affect preservative performance in formulation. These factors include other formulation components, product pH, product A_w, the intended shelf life of the product, and processing and storage conditions (Orth *et al.* 2006).

Although there are over 50 preservatives available for use in HPC products, the most frequently used preservatives over the last 20-25 years have belonged to three classes: the parabens, the formaldehyde releasing agents, and the isothiazolinones (Lundov *et al.* 2009). These classes are desirable as they generally have broad antimicrobial activity and are stable over a wide pH range (Lundov *et al.* 2009). Examples of commonly used preservative classes are given in section 1.1.5, along with examples of specific compounds in Table 1.2. Even when efficacious, a single preservative may be insufficient to protect complex formulations from contamination and spoilage (Orth *et al.* 2006). Combinations of preservatives, often with different modes of action, are therefore used to boost the spectrum of activity that can be achieved (Orus and Leranoz 2005). Using combinations can lower the overall concentrations of individual preservatives, which lessens consumer exposure and minimises the emergence of resistant or tolerant organisms (Orth *et al.* 2006).

In addition, certain combinations of preservatives may exhibit synergy, whereby the activity of the combination is greater than would be expected from the simple addition of their individual activities (Denyer *et al.* 1985). An example of a commercially available preservative combination demonstrating synergistic activity is Phenonip, a mixture of phenoxyethanol and parabens (Parker *et al.* 1968). Chemical compounds, known as preservative potentiators or

preservative enhancers, are also incorporated into products to increase the antimicrobial activity of preservatives (Orth *et al.* 2006). These compounds are not regulated in the same way as preservatives and are discussed further in Section 1.1.5.7. Furthermore, other factors such as pH and A_w affect the ability of a product to support microbial growth and are taken into account when evaluating a preservative system (Orth *et al.* 2006). In recent years there has been an increased consumer demand for 'preservative free' or 'natural' products which has challenged the personal care industry to use milder preservation systems or look at 'self-preserving' formulations (Orth *et al.* 2006; Varvaresou *et al.* 2009). A combination of strategies have been assessed, including the use of 'Hurdle Technology' principles and alternative or natural preservatives, often derived from plants, which are not under the same regulations as classical preservatives (Varvaresou *et al.* 2009).

Preservatives are regulated for use in personal care products, with different regulations in different regions. The European Communities Cosmetic Directive, the Ministry of Health and Welfare and the FDA, regulate or restrict the use of preservatives in Europe, Japan and the United States, respectively. The legislation in Europe is outlined in the Council Directive 76/768/EC, within which annex II to VII specify prohibited or approved preservatives, along with their maximum in-use concentrations (Lundov *et al.* 2009). Changes to the content of the annexes are based on recommendations from the SCCP, taking into account consumer safety and adhering to the microbiological quality expectations of finished products (Lundov *et al.* 2009). In Japan, where the regulations are most stringent, the fewest numbers of preservatives are permitted for use in products (Orth *et al.* 2006), and lists of approved and banned substances are published by the Ministry of Health and Welfare under the Pharmaceutical Affairs Law (Chisvert and Salvador 2011). The situation is different in the United States, as there are no lists of approved preservatives, only a short list of substances banned or restricted by the FDA (Chisvert and Salvador 2011). Due to the varying restrictions, it is very difficult for a company to develop a preservative system that can be implemented in product formulations globally (Orth *et al.* 2006). Regulations are in place to ensure that consumers are not at risk from preservative exposure, which has the potential to cause contact dermatitis if the preservatives are skin sensitizers or irritants. In addition, other harmful activities are assessed, for example the oestrogenic activity that has been associated with parabens (Harvey and Everett 2004; Orth *et al.* 2006).

1.1.5 Key preservatives in HPC products

The following sections will introduce preservatives and preservative enhancing agents of importance to the home and personal care industry, highlighting which compounds were used in this study. A summary of preservative compounds and preservative enhancing agents is given in Table 1.2.

1.1.5.1 Parabens

Parabens are alkyl esters of para-hydroxybenzoic acid have been one of the most widely used groups of preservatives in the personal care industry (Soni *et al.* 2001). The predominant forms used in products include the methyl, n-propyl and the n-butyl esters (Table 1.2), which can be added individually or in combination, supplied by a range of manufacturers (Brannan 1997). Properties of parabens contributing to their utility as preservatives include a broad spectrum of antimicrobial activity, stability over a wide pH range and at high temperatures, sufficient solubility in water to achieve effective concentrations in products, and a low toxicity (Soni *et al.* 2001). The antimicrobial activity of parabens increases as the chain length of the ester group increases, but this also decreases the water solubility, meaning that the shorter chain length esters are more commonly used in products (Soni *et al.* 2005). Parabens have higher activity against fungi than bacteria and are more active against Gram-positive than Gram-negative bacteria (Soni *et al.* 2005). They are primarily membrane active agents and disrupt the proton motive force of the bacterial cellular membrane, which can lead to the acidification of the cytoplasm (Lambert 2008). Other effects include the leakage of intracellular components and the inhibition of amino and oxo acid uptake (Brannan 1997; Maillard 2002). Although parabens are easy to formulate with and well tolerated, concerns over their safety to consumers have arisen in the last two decades, which had led to changes to their use in Europe. Studies have reported that parabens have been found in human breast tumours and may have oestrogenic potential (Harvey and Everett 2004). Whilst the link is still equivocal, amendments to the EU Cosmetics Directive have been made to prevent the use of long chain parabens isopropylparaben, isobutylparaben, phenylparaben, benzylparaben and pentylparaben (Commission regulation (EU) No 1004/2014, made on September 18th 2014), with the use of other parabens regulated at 0.4% (singly) or 0.8% (as a mixture).

1.1.5.2 Formaldehyde and formaldehyde releasing agents

The use of free formaldehyde as a preservative is decreasing, instead organic compounds are incorporated into products which hydrolyse gradually to release low levels of formaldehyde (Brannan 1997). Different formaldehyde releasing agents release varying amounts of free formaldehyde into products (Brannan 1997). For the agent dimethyloldimethyl hydantoin (DMDMH; trade name Glydant, manufactured by Lonza; Table 1.2), the rate of release of formaldehyde is pH dependent, with higher rates at an increased pH (9-10.5), compared to a lower pH (3-5) (Lambert 2008). The broad spectrum antimicrobial activity of formaldehyde is attributed to the overt reactivity of the molecule. Formaldehyde targets proteins, DNA and RNA, reacting with several sites of importance on the biomolecules. Alkylation of amino, imino, amide, carboxyl and thiol groups of proteins, and the ring nitrogen atoms of purine bases may occur, which can result in the formation of intermolecular cross-linkages (Brannan 1997; Denyer and Stewart 1998). Formaldehyde has been shown to be an irritant, a moderate to strong allergen, and can be a potent sensitiser, with reported sensitisation rates between 1% and 9% (Sasseville 2004). The concentrations of these preservatives are regulated in the EU, with free formaldehyde being permitted at either 0.1% (for oral hygiene) or 0.2% (except oral hygiene), and 0.6% for the formaldehyde releasing agent dimethylol dimethyl hydantoin (DMDMH).

1.1.5.3 Isothiazolinones

Isothiazolinones are widely used in a variety of applications including cooling water, paper and textiles, in addition to home and personal care products (Laopaiboon *et al.* 2001). Suspensions of isothiazolinones, rather than pure compounds, are available commercially (Lambert 2008), which provide adequate preservation at low levels and have a broad spectrum of antimicrobial activity (Brannan 1997). The predominant mechanism of action of the isothiazolinones is the oxidation of thiol groups, found on enzymes and other proteins, which are essential for function. Progressive loss of protein thiols disrupts pathways critical for cell metabolism and generates free radicals which further damage the cell (Williams 2007). Chlorinated isothiazolinones may also interact with non-thiol containing amino acids and interfere with DNA replication (Collier *et al.* 1990). Commercially available preservatives include methylisothiazolinone (MIT), chloromethylisothiazolinone (CITMIT), benzisothiazolinone (BIT), and a blend known as Kathon CG which is a registered trademark of the company Rohm and Haas. Kathon CG contains CIT and MIT in the ratio 3:1, respectively, and was first introduced used in Europe in the

cosmetics industry in the mid-1970s (Sasseville 2004). The isothiazolinones are known to cause contact dermatitis and are sensitising agents. Focussing on CITMIT, the prevalence of contact allergy in Europe has been found to fall between 2-2.5% (Lundov *et al.* 2009). In the EU only MIT and CITMIT are permitted for use in personal care products, at the concentrations 0.01% and 0.0015% respectively.

1.1.5.4 Biguanides

The biguanide biocide chlorhexidine (Table 1.2) is widely used in antiseptic products, as a disinfectant and as a preservative (McDonnell and Russell 1999). It has a broad spectrum of antimicrobial activity being active against both Gram-positive and Gram-negative bacteria, low toxicity and low irritancy (Moore and Payne 2008). The activity of biguanides is pH dependent, generally between pH 3-9, with the range being narrower for chlorhexidine, between pH 5-7 (Owen 2006). Chlorhexidine is a membrane active agent and damages the cell wall, promoting its uptake into the cytoplasm (Maillard 2002). At lower concentrations chlorhexidine inhibits membrane-associated enzymes and leads to leakage of cellular components, whilst at higher concentrations coagulation of cytoplasmic constituents is observed (Moore and Payne 2008).

1.1.5.5 Alcohols

Several alcohols, including ethanol, phenethyl alcohol, benzyl alcohol and phenoxyethanol are used as preservatives in personal care products, with phenoxyethanol being used more frequently in recent times (Brannan 1997). Phenoxyethanol (Table 1.2) is compatible with most raw materials, is easy to formulate in the aqueous phase, can withstand temperatures up to 85°C and has activity between PH 3 and 10 (Krowka *et al.* 2014). Whilst not as efficacious as other preservatives such as the parabens, phenoxyethanol has good activity against Gram-negative bacterial species including *P. aeruginosa*, and may be used alone or in combination in products (Orth *et al.* 2006; Lambert 2008). Phenoxyethanol is a membrane active agent, disrupting cellular membranes and causing the leakage of intracellular components (Maillard 2002). In addition, some evidence has shown that phenoxyethanol inhibits enzymes involved in the tricarboxylic acid pathway (Gilbert *et al.* 1977). The low sensitisation potential of phenoxyethanol has likely expanded its use in personal care products (Krowka *et al.* 2014) and it is regulated to be used at a concentration of 1% in the EU.

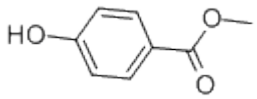
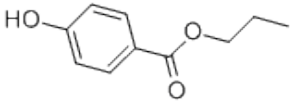
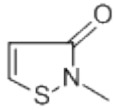
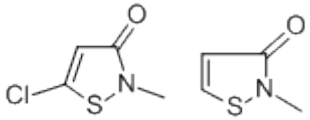
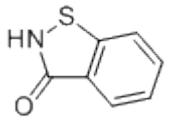
1.1.5.6 Organic acids

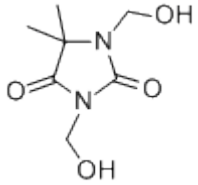
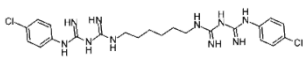
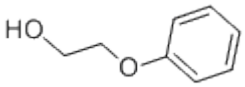
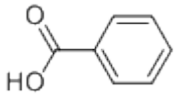
Organic acids are becoming increasingly popular for use as preservatives in personal care products. The undissociated form of the acid is responsible for the antimicrobial activity which means that for effective preservation, the pH of the product must below 5 to prevent transformation of the acid into its salt (Brannan 1997). Benzoic acid (Table 1.2) is one of the longest-used chemical preservatives and has been used in a variety of industries such as the food, cosmetics and drug industries (Chipley 1983). Properties that make benzoic acid a useful preservative include a low cost, ease of incorporation in to products and low toxicity (Chipley 1983). The mechanism of action of benzoic acid is similar to the parabens, with both being membrane active agents. Benzoic acid targets the proton motive force which leads to leakage of intracellular contents and acidification of the cytoplasm (Maillard 2002).

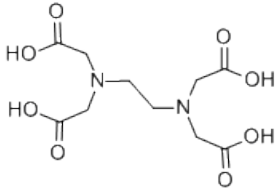
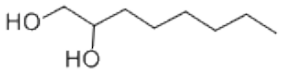
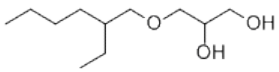
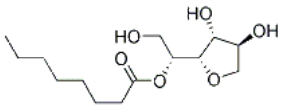
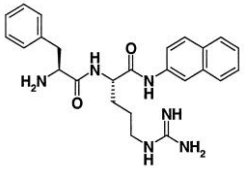
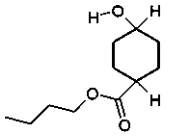
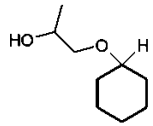
1.1.5.7 Preservative enhancing agents

Preservative enhancing agents, or 'preservative potentiators' can increase the antimicrobial efficacy of the preservative without having to increase the preservative concentration, and are not under the same legislation as preservatives (Orth et al. 2006). Chelating agents, such as ethylenediamine tetracetic acid (EDTA), can potentiate the activity of many other antimicrobial agents, including preservatives. EDTA has greatest impact on Gram-negative bacteria and removes divalent cations (Mg^{2+} and Ca^{2+}) from the outer membrane, increasing the permeability of the cell (Brannan 1997). Polar compounds which resemble the phospholipid molecules within the cell membrane bilayer have also been used to boost preservative activity by interference with the cell membrane (Papageorgiou et al. 2010). Examples of such compounds include caprylyl glycol, ethylhexylglycerin and sorbitan caprylate, marketed under different companies (Table 1.2). This PhD evaluated two cyclohexyloxyl derivative compounds (CX3 and CX7; Table 1.2) and an efflux pump inhibitor (phenyl-arginine-beta-naphthylamide; PAβN) as novel preservative boosters (see Chapter 6). Whilst no previous studies have reported on CX3 and CX7, there is evidence that PAβN permeabilises the outer membrane of Gram-negative bacteria to certain antibiotics (Lamers *et al.* 2013) and therefore has potential to enhance preservative efficacy.

Table 1.2 Preservatives and preservative enhancing agents used in the HPC industry and in this study

		Chemical compound(s)	Structure	EU regulated level[1]	Mechanism of action (reference)	Trade name (% active ingredient)	Manufacturer
Preservatives	Parabens	Methyl paraben		0.4% (one paraben) 0.8% (paraben mixtures)	Parabens are membrane active agents and disrupt the proton motive force, leading to acidification of the cell interior (Lambert 2008).	Nipagin®M (100%)	Clariant
		Propyl paraben		0.4% (one paraben) 0.8% (paraben mixtures)		Nipasol®M (100%)	Clariant
	Isothiazolinones	Methylisothiazolinone (MIT)[2]		0.01%	Isothiazolinones are oxidising agents and interact strongly with thiol groups (R-SH) which are vital to the function of cytoplasmic and membrane bound enzymes (Denyer and Stewart 1998). The disruption of cellular pathways also leads to the production of free radicals, and the inhibition of ATP synthesis and utilisation (Williams 2007) . Chlorinated forms may also interact with non-thiol containing amino acids and inhibit DNA replication (Collier *et al.* 1990)	Neolone M10 (9.7%)	Rohm & Haas
		Chloromethylisothiazolinone and Methylisothiazolinone blend in the ratio 3:1 (CITMIT)[2]		0.0015%		Kathon CG (1.5%)	Rohm & Haas
		Benzisothiazolinone (BIT)[2]		Not permitted in the EU		Koralone B-120 (20%)	Rohm & Haas

	Formaldehyde releasing agents	Dimethylol dimethyl hydantoin (DMDMH)[2]		0.6%	Formaldehyde is a highly reactive chemical and acts as a mutagen and an alkylating agent. Interaction with proteins and nucleic acids containing amino, imino, amide, carboxyl and thiol groups, can result in intermolecular crosslinking (Denyer and Stewart 1998).	Glydant (55%)	Lonza
	Biguanides	Chlorhexidine (CHX)[2]		0.3%	Chlorhexidine is a membrane active agent which disrupts the cytoplasmic membrane leading to leakage of intracellular components, and at high concentrations causes coagulation of the cytosol (Maillard 2002)	Chlorhexidine digluconate	Sigma-Aldrich
	Alcohols	Phenoxyethanol (PHE)[2]		1.0%	Phenoxyethanol is a membrane active agent and causes generalised loss of cytoplasmic membrane function and changes in outer membrane structure, leading to leakage of intracellular constituents (Brannan 1997; Maillard 2002)	Phenoxetol (100%)	Clariant
	Organic acids	Benzoic acid (BA)[2]		2.5% (rinse off) 1.7% (oral care) 0.5% (leave-on)	Organic acids are membrane active agents. In addition to disrupting the proton motive force, which leads to acidification of the cell interior, organic acids also cause the leakage of intracellular components (Maillard 2002)	-	Sigma-Aldrich

Preservative enhancing agents	Chelating agents	Ethylenediaminetetraacetic acid (EDTA)		-	EDTA chelates Mg^{2+} and Ca^{2+} ions from the Gram-negative bacterial outer membrane, disrupts membrane integrity and increases permeability to antimicrobial agents (Vaara 1992)	-	Sigma-Aldrich
	Membrane disruptors	Caprylyl glycol (also known as 1, 2-Octanediol)		-	These molecules have surfactant properties, being composed of a hydrophilic head region and a hydrophobic tail region, and affect the interfacial tension at the cell membrane, aiding preservative penetration (Varvaresou *et al.* 2009; Papageorgiou *et al.* 2010; Pilz 2012)	Dermosoft®Octiol	Dr. Straetmans
		Ethylhexylglycerin		-		Sensiva® SC 50	Schülke
		Sorbitan caprylate		-		Velsan SC	Clariant
	Efflux pump inhibitors[3]	Phenyl-arginine-beta-naphthylamide (PAβN)[2]		-	PAβN is an efflux pump inhibitor which inhibits multiple Gram-negative RND-family transporters by competitive inhibition of substrate binding sites (Okandeji *et al.* 2011)	-	Sigma-Aldrich
	Novel compounds for evaluation (cyclohexyloxyl derivatives)[3]	CX-3[2]		-	Mechanism of action unknown	CX-3 (96%)	Merck
		CX-7[2]		-		CX-7 (100%)	Merck

Footnotes: [1]EU Cosmetics Directive 76/768/EEC maximum regulated levels of preservatives; [2]Preservatives and preservative enhancing agents used in this study; [3]Not currently used as preservative enhancers, only evaluated by this study

1.2 Pseudomonas aeruginosa

1.2.1 *P. aeruginosa* as an opportunistic pathogen and industrial contaminant

P. aeruginosa is an extremely versatile microorganism with the ability to survive in diverse habitats including soil, water, plant and animal tissues, community and hospital environments (Morrison and Wenzel 1984; Stover *et al.* 2000; Kerr and Snelling 2009). First isolated in 1882 by the French pharmacist Carle Gessard from an infected surgical wound (Gessard 1882), *P. aeruginosa* has subsequently been recognised as an important opportunistic pathogen in humans. A broad spectrum of illness may be caused by *P. aeruginosa* including pneumonia, urinary tract infections, wound and burn infections, bacteraemia and septicaemia (Kerr and Snelling 2009). The majority of these infections are encountered in healthcare settings and are rare in healthy individuals. *P. aeruginosa* is the major pathogen in cystic fibrosis patients and is responsible for chronic, life-threatening pulmonary infections which are extremely difficult to eradicate (Gomez and Prince 2007). Of great concern is the limited number of effective anti-pseudomonal drugs and increasing rates of antimicrobial resistance amongst clinical isolates (Rossolini and Mantengoli 2005).

Many factors contribute to the widespread prevalence of *P. aeruginosa* and its success as an opportunistic pathogen. Although considered an obligate aerobe, *P. aeruginosa* can exhibit anaerobic metabolism in the presence of nitrates or the amino acid arginine. Anaerobic metabolism may enhance the survival of *P. aeruginosa* in the soil or in certain animal tissues where molecular oxygen is scarce (Vasil 1986). *P. aeruginosa* also has minimal nutritional requirements, exemplified by its ability to grow in distilled water (Favero *et al.* 1971; Kayser *et al.* 1975), and displays high levels of metabolic versatility, being able to utilise over 75 organic compounds for growth (Stanier *et al.* 1966). In addition, *P. aeruginosa* can withstand a wide temperature range of between 20°C and 42°C, with an optimum of 37°C (Morrison and Wenzel 1984). Tolerance for many different antimicrobial agents (Kerr and Snelling 2009) also permits the persistence of *P. aeruginosa* in varied environments. In the compromised host, a multitude of virulence factors possessed by *P. aeruginosa* promote successful infection. Regulation of these factors is efficient and largely by means of quorum sensing, a form of cell-to-cell communication. Quorum sensing pathways are also important in the formation of biofilms, both on living and abiotic surfaces, which offer *P. aeruginosa* protection from extraneous stresses and facilitate the sequestration of nutrients (Wagner *et al.* 2008). Notably, pigment production is characteristic of *P. aeruginosa* and can be used as a means for its identification. Pyocyanin, the blue-green phenazine pigment (Vasil 1986), is exclusively produced by some, but

not all, *P. aeruginosa* strains (Haynes 1951) and can be considered a virulence factor as it plays a role in infection (Lau *et al.* 2004).

To date, the focus of *P. aeruginosa* research has been the pathogenesis, epidemiology and treatment of infections caused by clinical strains. Outside of healthcare settings in the natural environment, *P. aeruginosa* populations have not been as thoroughly investigated (Lavenir *et al.* 2007). Even less is known about *P. aeruginosa* in industrial settings, despite it being a problem contaminant of raw materials, pharmaceutical and cosmetic products (Jimenez 2007)(Figure 1.1), and other industrial products such as fuels (Edmonds and Cooney 1967; White *et al.* 2011). Contamination is a major cause of product recalls worldwide and, more seriously, represents a threat to the health of consumers (Jimenez 2001a). In order to begin to understand *P. aeruginosa* in an industrial context, isolates from contaminated products need to be identified, the diversity of strains associated with contamination surveyed, and the mechanisms which permit the survival of *P. aeruginosa* in industrial formulations elucidated.

1.2.2 Methods for the detection and identification of *P. aeruginosa*

The routine detection of bacterial species in medical and industrial settings is largely still via culture-dependent techniques (Orus and Leranoz 2005; van Belkum *et al.* 2013), which are relatively inexpensive, generally reliable and identify most of the commonly encountered bacteria (Woo *et al.* 2008; Deschaght *et al.* 2011). In the case of *P. aeruginosa*, numerous selective agars and broths have been developed for its isolation, most of which exploit the tolerance of *P. aeruginosa* to antimicrobial agents and generally inhibit the growth of other bacteria. Media using positive selection strategies have also been described, such as those containing acetamide (Hedberg 1969; Smith and Dayton 1972; Szita *et al.* 1998; Szita *et al.* 2007) and asparagine (Favero *et al.* 1971) both of which *P. aeruginosa* can use as the sole source of carbon and nitrogen. More recently a chromogenic media for the detection of *P. aeruginosa* has been developed, which claims to be selective and differential for simultaneous detection and identification (Laine *et al.* 2009). A summary of selective media for *P. aeruginosa* is given in Table 1.3. Whilst production of pyocyanin can unequivocally identify *P. aeruginosa*, this pigment is not produced by all strains under different conditions (Smirnov and Kiprianova 1990). In these cases, and when selectivity of the medium is doubted, further diagnostic tests can be performed to confirm the isolation of *P. aeruginosa* (Phillips 1969). In addition, commercial kits are available for the identification of bacteria such as the API identification schemes (Biomerieux, Les Halles, France) and the automated Vitec® (Biomerieux) system, which assess several different biochemical tests simultaneously, can also be used for bacterial identification.

Genotypic identification methods have a number of advantages over culture-dependent techniques including that they circumvent the problem of phenotypic variability under different growth conditions. This is especially relevant in the case of *P. aeruginosa* which can display variable colony morphology and levels of pigment production which may confound phenotypic identification (Spilker *et al.* 2004). In addition, selective culture media may not always have sufficient selectivity and have been demonstrated to misidentify non-*P. aeruginosa* fluorescent pseudomonads from environmental (river water) samples as *P. aeruginosa* (Pirnay *et al.* 2005). PCR and qPCR formats, including both SYBR Green-based qPCR (Jaffe *et al.* 2001; Qin *et al.* 2003; Motoshima *et al.* 2007; Choi *et al.* 2013) and hydrolysis probe-based qPCR (Anuj *et al.* 2009), have been developed for the detection of *P. aeruginosa.* To date these formats have targeted ten different genes/regions, attaining species-specificity through choice of primer sequences (Table 1.4). As can be seen from Table 1.4, the primers pairs evaluated by multiple studies have high specificity (except for the *fliC* primer pair) and sensitivity. These PCRs have been applied to DNA extracted from pure *P. aeruginosa* cultures in addition to clinical and environmental samples (Deschaght *et al.* 2011). There is opportunity for the design of improved PCRs for *P. aeruginosa*, however, through the utilisation of the increasing number of genome sequences available for the species (Choi *et al.* 2013).

In addition to the application of species-specific PCRs to the detection of bacteria, 16S rRNA gene sequencing can be performed to identify bacterial species. This type of analysis may be used to provide species identification for cultivated isolates (Janda and Abbott 2007; Woo *et al.* 2008), or applied to DNA extracted directly from samples, also giving an idea of the total diversity present (Salipante *et al.* 2013; Dickson *et al.* 2014). Studies comparing conventional/commercial tests examining phenotypic characteristics to 16S rRNA gene sequence data for cultivated isolates have determined that 16S rRNA gene analysis facilitated the identification of an increased number of species and had identification rates between 62-91% (Janda and Abbott 2007). Studies utilising next-generation technologies to perform deep sequencing of 16S rRNA genes in clinical samples such as sputum (Salipante *et al.* 2013) and bronchoalveolar lavage fluid (Dickson *et al.* 2014) found that in general the results of conventional culture and sequencing were concordant, although sequencing facilitated the identification of a greater number of species than culture. There is therefore the potential for 16S rRNA gene sequencing to be used in diagnostic microbiology but attention is needed to ensure correct data interpretation (Woo *et al.* 2008). Furthermore, the limited phylogenetic resolution that can be achieved with the 16S rRNA gene (Janda and Abbott 2007) and the limitations of DNA extraction techniques have to be considered (Deschaght *et al.* 2011).

Table 1.3 Selective agents and media for the culture-dependent isolation of *Pseudomonas aeruginosa*

Selective agents	Concentration (g/L)	Name of medium	Comment	Reference
Individual selective agents				
Triclosan	0.025	Pseudomonas Isolation Agar (PIA) [Sigma-Aldrich; BD Difco™]	Modified from Kings Medium A* (King *et al.* 1954) by the addition of triclosan; triclosan also termed Irgasan in PIA	-
Cetrimide	1.0	Cetrimide agar	'Old' form of cetrimide in nutrient agar	(Lowbury 1951)
	0.3	Cetrimide agar	'New' form of cetrimide (cetavlon) in nutrient agar, lower concentration required for sufficient selectivity than the 'old' form	(Lowbury and Collins 1955)
		Cetrimide agar	Cetrimide (cetavlon) in Kings medium B** (King *et al.* 1954)	(Brown and Lowbury 1965)
		Pseudosel Agar [BD BBL™]	Modified from Kings medium A (King *et al.* 1954) by the addition of cetrimide	-
		Pseudomonas Cetrimide Agar [Oxoid Ltd.]	Modified from Brown and Lowbury (1965)	-
Asparagine	3.0	Asparagine broth	Developed for the isolation of *P. aeruginosa* from water samples	(Favero *et al.* 1971)
Acetamide	10	Acetamide medium	Modified from Christensen (1946). Peptone included as carbon source, acetamide as nitrogen source.	(Bühlmann *et al.* 1961)
	20	Acetamide agar	Acetamide as carbon and nitrogen source	(Hedberg 1969)
		Acetamide broth	Modified from Hedburg (1969). Acetamide as carbon and nitrogen source	(Smith and Dayton 1972)
	4.0	Z-Agar	Developed for the isolation of *P. aeruginosa* from food and water	(Szita *et al.* 1998)
	5.0	Z-broth		
Nitrofurantoin	0.05	Nitrofurantoin agar/both	-	(Thom *et al.* 1971)
C-390 (9-chloro-9-(4-diethylaminophenyl) -10-phenylacridan hydrochloride)	0.03	-	C-390 in Kings Medium B (agar)	(Marold *et al.* 1981)
		-	C-390 in Brain heart Infusion broth and Mueller-Hinton agar	(Fader *et al.* 1988)
	0.001-0.05	-	Range of C-390 concentrations evaluated,≥0.015 g/L optimum. Different base media also looked at	(Davis *et al.* 1983)
	0.05-0.1	-	Range of C-390 concentrations in brain heart infusion broth evaluated, ≥0.025 g/L optimum. Claims to identify *P. aeruginosa* within 4 hours	(Araj 1984)
Cadmium chloride	-	-	-	(Drake 1966)
Combinations of selective agents				
Cetrimide (C) Nalidixic acid (N)	0.2 (C) 0.015 (N)	Nalidixic acid cetrimide agar	-	(Goto and Enomoto 1970)
	0.2 (C) 0.015 (N)	Cetrimide-nalidixic acid agar	Cetrimide-nalidixic acid agar (formulation of Goto and Enmoto, 1970) evaluated and found to be superior to cetrimide agar of Brown and Lowbury (1965)	(Lilly and Lowbury 1972)
	0.2 (C) 0.015(N)	Pseudomonas Agar Base with C-N supplement [Oxoid Ltd]	C-N supplement has selective agents in the same concentrations as those of Goto and Enmoto (1970) and Lilly and Lowbury (1972)	-
C-390 Phenanthroline (P)	0.03 (C-390) 0.03 (P)	PC Agar	PC agar superior to media containing only C-390 as selective agent. Phenanthroline alone not selective for *P. aeruginosa* but in PC agar suppressed the growth of non-*P. aeruginosa* stains	(Campbell *et al.* 1988)
Kanamycin (K) Nalidixic acid (N) Sulfapyridine (S)	0.008 (K) 0.037 (N) 0.176 (S)	mPA Agar	Developed for the isolation and enumeration of *P. aeruginosa* from water samples using a membrane filtration procedure	(Levin and Cabelli 1972)
		mPA-B Agar		(Dutka and Kwan 1977)
	0.008 (K) 0.037 (N)	mPA-C Agar		(Brodsky and Ciebin 1978)

Table 1.4 Detection of *Pseudomonas aeruginosa* using PCR-based methods: target genes and primer pairs

Gene	Primers	Primer sequence (5'→ 3')	Product size (bp)	Specificity/sensitivity (%)	Reference
16S rRNA	PA-SS-F	GGGGGATCTTCGGACCTCA	956	100/100	(Spilker *et al.* 2004)[a]
	PA-SS-R	TCCTTAGAGTGCCCACCCG		94/100	(Lavenir *et al.* 2007)
16S-23S rRNA ITS	PA1	TCCAAACAATCGTCGAAAGC	181	100/100	(Lavenir *et al.* 2007)[b]
	PA2	CCGAAAATTCGCGCTTGAAC			
oprI	OPR1	GCTCTGGCTCTGGCTGCT	197	99/98	(Qin *et al.* 2003)
	OPR2	AGGGCACGCTCGTTAGCC		80/100	(Lavenir *et al.* 2007)
oprL	PAL1	ATGGAAATGCTGAAATTCGGC	504	100/100	(De Vos *et al.* 1997)[a]
				86/100	(Anuj *et al.* 2009)
	PAL2	CTTCTTCAGCTCGACGCGACG		100/100	(Jaffe *et al.* 2001)
				86/100	(Lavenir *et al.* 2007)
fliC	Fla1	GCCTGCAGATCGCCAACC	1000/1300	13/100	(Lavenir *et al.* 2007)[c]
	Fla2	GGCAGCTGGTTGGCCTG			
ecfX	ECF1	ATGGATGAGCGCTTCCGTG	528	100/100	(Lavenir *et al.* 2007)[a]
	ECF2	TCATCCTTCGCCTCCCTG		100/0	(Choi *et al.* 2013)
	ecfX-F	CGCATGCCTATCAGGCGTT	63	100/100	(Anuj *et al.* 2009)[a]
	ecfX-R	GAACTGCCCAGGTGCTTGC			
gyrB	GyrPA-398	CCTGACCATCCGTCGCCACAAC	222	100/100	(Anuj *et al.* 2009)
				100/100	(Qin *et al.* 2003)[a]
	GyrPA-620	CGCAGCAGGATGCCGACGCC		100/98	(Motoshima *et al.* 2007)
				100/100	(Lavenir *et al.* 2007)
				70 (approx.)/100	(Choi *et al.* 2013)
ecfX + *gyrB*	ecfX-F	CGCATGCCTATCAGGCGTT	63	100/100	(Anuj *et al.* 2009)
	ecfX-R	GAACTGCCCAGGTGCTTGC			
	GyrPA-398	CCTGACCATCCGTCGCCACAAC	222		
	GyrPA-620	CGCAGCAGGATGCCGACGCC			
toxA (Exotoxin A)	ETA1	GACAACGCCCTCAGCATCACCAGC	396	>95/>95	(Khan and Cerniglia 1994)[a]
				100/94	(Qin *et al.* 2003)
	ETA2	CGCTGGCCCATTCGCTCCAGCGCT		100/95	(Lavenir *et al.* 2007)
				100/98	(Anuj *et al.* 2009)
algD GDP mannose	VIC-1	TTCCCTCGCAGAGAAAACATC	520	100/89	(Qin *et al.* 2003)
	VIC-2	CCTGGTTGATCAGGTCGATCT		100/100;100/100	(da Silva Filho *et al.* 1999)[a] (da Silva Filho *et al.* 2004)
O-antigen acetylase PA431CF		CTGGGTCGAAAGGTGGTTGTTATC	232	100/100	(Choi *et al.* 2013)

Footnotes: [a]First study to describe and evaluate particular primer pair; [b]Primers first described by Tyler *et al.* (Tyler *et al.* 1995); [c]Primers first described by Spangenberg *et al.* (Spangenberg *et al.* 1996)

1.2.3 *P. aeruginosa* genotyping, diversity and population structure

DNA-based typing systems (Table 1.5) are invaluable tools in epidemiological investigations and many different methods have been developed for typing below the species level. From a clinical perspective genetic typing is extremely useful, particularly in outbreak situations, as it allows questions to be answered concerning the source and route of transmission of pathogens. In addition to this, typing can provide information about the clonality of a pathogen and identify hyper-virulent strains (Olive and Bean 1999). Strain typing also may be of use in industry when dealing with bacterial contamination (White *et al.* 2011). For example, the application of genetic typing techniques in industrial settings could determine whether contaminating isolates share a common genotype and if contamination is occurring from a common source, such as the water supply. With regards to *P. aeruginosa*, genetic typing methods have been used extensively to determine the epidemiology of *P. aeruginosa* infection, notably infections contracted by cystic fibrosis patients (van Mansfeld *et al.* 2010; Fothergill *et al.* 2012). Typing methods are also useful when applied to the broader investigation of *P. aeruginosa* diversity and population biology.

The population structure of *P. aeruginosa* has been the subject of investigations and the current general consensus is that of a non-clonal epidemic structure (Pirnay *et al.* 2009; Dettman *et al.* 2013). From these analyses, it appears that isolates from different environments are genotypically and functionally indistinguishable from one another (Nicas and Iglewski 1986; Römling *et al.* 1994; Rahme *et al.* 1995; Foght *et al.* 1996; Alonso *et al.* 1999), and different strains exhibit no clear selection for a particular habitat (Pirnay *et al.* 2009), demonstrated by globally distributed clonal types such as clones C and PA14 (Tümmler *et al.* 2014). However, evidence which contends this exists, complicating the consensus model and includes the association of multi-drug resistant clones with the nosocomial environment (Pirnay *et al.* 2009), transmissible *P. aeruginosa* clones within cystic fibrosis patients (Lanotte *et al.* 2004; van Mansfeld *et al.* 2010), a cluster of strains associated with corneal infections (Stewart *et al.* 2011), and certain clonal complexes with river systems (Selezska *et al.* 2012) or the open ocean (Khan *et al.* 2008). This suggests that the population is made up of epidemic clonal types that are generally ubiquitous, in addition to niche specialists (Tümmler *et al.* 2014). With the exception of a small number of studies (Pirnay *et al.* 2005; Khan *et al.* 2008; Selezska *et al.* 2012) *P. aeruginosa* research has largely had a clinical focus. Little is known about *P. aeruginosa* populations outside of the nosocomial environment (Lavenir *et al.* 2007), as even those studies including environmental isolates in their collections have done so in relatively small proportions (10-25%), and mainly considered the clinical implications of their findings

(Selezska *et al.* 2012). The diversity of *P. aeruginosa* strains in industrial settings and relation to overall population biology of the species is as of yet unknown.

Table 1.5 Genotyping methods which have been used for *Pseudomonas aeruginosa*

Typing method	Description of method	Comments	References
Fingerprinting methods – strain-specific banding patterns resulting from the electrophoretic separation of DNA fragments			
Pulsed-Field Gel Electrophoresis (PFGE)	Bacterial cells embedded within agarose plugs are lysed and the bacterial chromosome digested within an infrequently cutting restriction enzyme. The digested plugs are inserted into an agarose gel and subjected to electrophoresis (PFGE). The polarity of the current in the PFGE apparatus is periodically alternated to allow the separation of large DNA fragments (10-800 kb). The resulting banding pattern can then be visualised.	Previously widely used and often considered to be the 'gold standard' typing method. Highly discriminatory but time-consuming, expensive and labour- intensive, and minor genetic changes (point mutations/insertions/deletions) may alter results	(Olive and Bean 1999; Syrmis *et al.* 2004; Fothergill *et al.* 2010)
Random Amplified Polymorphic DNA (RAPD) typing	RAPD (also known as arbitrarily primed-PCR) uses short primers (approximately 10 bp) of arbitrary sequence which bind, at low stringency conditions, to complementary sequences distributed around the bacterial chromosome. If two primers anneal in the correction orientation and within a few kilobases of each other, PCR amplification will occur. This results in DNA fragments of varying sizes which can be separated by electrophoresis and visualised.	RAPD is very discriminatory, fast and inexpensive. The RAPD process is sensitive to changes in PCR conditions (particularly the annealing temperature), however, which may affect the binding of the primers leading to variability in banding patterns. In addition, RAPD protocols must be optimised for different species using different primers and reaction conditions making standardisation of this approach difficult. The RAPD 272 primers have been demonstrated to have high discriminatory power for use in the strain typing of *P. aeruginosa*.	(Mahenthiralingam *et al.* 1996; Olive and Bean 1999)
Repetitive – element PCRs (BOX and ERIC-PCR)	Rep-PCR assays target highly conserved repetitive sequence elements distributed throughout bacterial genomes. Two groups of these elements have been used in the strain typing of *P. aeruginosa*: enterobacterial repetitive intergenic consensus (ERIC) sequences and BOX elements. Using primers which are complementary to either ERIC or BOX elements, DNA sequences of different lengths between the elements can be amplified, separated by electrophoresis and visualised.	Rep-PCRs are inexpensive and fast. They target conserved repetitive elements and use highly stringent conditions so are stable and relatively easily standardised. Rep-PCRs are reported to have good concordance with PFGE results but generally have slightly less discriminatory power.	(Versalovic *et al.* 1991; Olive and Bean 1999; Syrmis *et al.* 2004)
Sequence-based methods			

Multilocus sequencing typing (MLST)	MLST indexes the variation of the sequences of several bacterial housekeeping genes. The genes included in an MLST scheme are different for different bacterial species. Fragments of the housekeeping genes are amplified using PCR and sequenced. For each housekeeping gene, distinct sequences are given an allele number, and the combination of different allele numbers defines the sequence type (ST) of a particular isolate. STs can be compared via an internet database.	As MLST uses sequence data it is an unambiguous and electronically portable typing technique. At present the costs of sequencing multiple genes for a large isolate collection are still relatively high. The MLST scheme for *P. aeruginosa* utilises the sequences of 7 housekeeping genes: *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*. A curated MLST database for *P. aeruginosa* has been established.	(Curran *et al.* 2004; Jolley *et al.* 2004; Khan *et al.* 2008)
Whole genome sequencing and bioinformatic analysis (e.g. using Ribosomal Multilocus Sequencing Typing [rMLST])	Genome sequencing and subsequent bioinformatic analyses can allow phylogeny and genetic relationships between genomes to be inferred. For example, rMLST is a recently described scheme which analyses the sequences of the 53 genes encoding the bacterial ribosomal protein subunits. As these genes are distributed throughout the chromosome whole genome sequences are needed for the analyses. rMLST has great potential for both taxonomic and typing analyses across all levels of bacterial diversity, and similarly to MLST, an expandable internet database has been established.	A complete genome sequence is the ultimate means to define the genotype of an isolate and cost of genome-sequencing is now becoming cost effective at approximately £50-200 per isolate, dependent on genome size.	(Jolley *et al.* 2012a)
Micro-probe array based			
Clondiag Array Tube	The Array Tube system probes both the core and accessory genome of *P. aeruginosa*. Labelled DNA from 58 targets distributed around the genome is generated directly from bacterial colonies by cycles of multiplex primer extension reactions. This DNA is then hybridised onto a microarray chip and detected colourimetrically to generate an electronically portable 58-binary marker genotype.	The Clondiag Array Tube process is rapid, inexpensive and robust. As the technique interrogates the accessory genome, in addition to the core genome, the presence of virulence markers and genomic islands can be identified. This technique has proved useful for identifying isolates of the Liverpool Epidemic Strain (LES) of *P. aeruginosa*.	(Wiehlmann *et al.* 2007b; Mohan *et al.* 2008; Fothergill *et al.* 2010)

1.2.4 *P. aeruginosa* genomics and evolution

The large genome of *P. aeruginosa* underpins its adaptive capabilities. Being between 5.2 and 7 Mbp (Schmidt *et al.* 1996), the *P. aeruginosa* genome is one of the largest observed for Gram-negative bacteria and has huge coding capacity and complexity. From the genome of *P. aeruginosa* PAO1, the first *P. aeruginosa* strain to be sequenced (Stover *et al.* 2000), it was seen that a relatively high proportion of genes were involved in regulation (over 8% of the genome), energy metabolism and transport of small molecules reflecting ecological flexibility. The size of the genome indicates genetic and functional complexity rather than gene duplication, with a large number of paralogous gene groups being identified (Silby *et al.* 2011). There are still over 2000 genes in the *P. aeruginosa* PAO1 genome of unknown function highlighting our limited insight into the full genetic repertoire of the species (Tümmler *et al.* 2014).

To date, over 1000 *P. aeruginosa* genomes have been sequenced, with many projects ongoing (Rumbaugh 2014). From this we have learnt that the 'core' genome of *P. aeruginosa* comprises approximately 4000 genes and is generally well conserved among clonal complexes, showing sequence diversities of 0.5-0.7% (Klockgether *et al.* 2011). The 'accessory' genome represents about 20% of the genome, is variable between strains, and is made up of 'regions of genomic plasticity' (RGP) associated with horizontal gene transfer (Klockgether *et al.* 2011; Tümmler *et al.* 2014). Analysis of the accessory genomes of sequenced isolates has identified many genes involved in metabolism, virulence and antibiotic resistance (Rumbaugh 2014), in addition to over 10,000 unique genes, hinting at a pan-genome that potentially exceeds 100,000 genes (Tümmler *et al.* 2014). *P. aeruginosa* has access to a broad gene pool as acquisition of foreign DNA into the RGP can be from other *P. aeruginosa* strains, Pseudomonads, or Gram-negative species such as *Escherichia coli* or *Salmonella enterica* (Pohl *et al.* 2014).

Advances in genome sequencing have facilitated large scale studies of genome evolution in *P. aeruginosa*. Chronic *P. aeruginosa* lung infections are observed in 60-70% of cystic fibrosis sufferers (Folkesson *et al.* 2012), with over 50% of cases harbouring the initially acquired clone long-term (Tümmler *et al.* 2014). This provides an opportunity to study genome evolution *in vivo* over decades. In addition to phenotypic adaptive traits, such as variation in colony morphology and alginate production, motility, virulence and antimicrobial resistance (Mowat *et al.* 2011), genomic changes are now being elucidated. Whilst no two studies have reported the same results, trends have emerged: (1) isolates are genotypically diverse, and mutations have been found in a range of genes (Feliziani *et al.* 2014); (2) hypermutability is observed, often to high levels, due to mutations in genes such as *mutS* and *mutL* involved in DNA mismatch repair (Marvig *et al.* 2013; Feliziani *et al.* 2014); (3) mutations in *mucA* are observed leading to

overproduction of alginate and conversion to the mucoid phenotype (Folkesson *et al.* 2012), and; (4) frequent mutation and loss of function of *lasR*, a transcriptional regulator of quorum sensing, which can decrease the expression of many virulence genes (Folkesson *et al.* 2012). Further work is required, however, to understand pathoadaptive mutations correlating with the evolutionary success of *P. aeruginosa* populations and sub-populations within the cystic fibrosis lung.

1.3 Bacterial resistance to antimicrobial agents with reference to P. aeruginosa

1.3.1 Antimicrobial agents: antibiotics and biocides

The resistance of *P. aeruginosa* to antimicrobial agents has been widely documented, although considerably more literature reports on antibiotic resistance than biocide resistance. At present, the antibiotic resistance of *P. aeruginosa* appears to be increasing and multidrug resistance is common in clinical settings. It is particularly worrying that no individual antibiotic is active against 100% of nosocomial *P. aeruginosa* isolates (Rossolini and Mantengoli 2005). This poses significant problems for the continued successful treatment of *P. aeruginosa* infections. The modes of action of antibiotic classes and are well defined and antibiotics generally have specific cellular targets. Resistance may be intrinsic, adaptive, or acquired, either through mutations in chromosomal genes, or via horizontal gene transfer of genes conferring a resistance phenotype (Tenover 2006).

Biocides (antiseptics, disinfectants and preservatives) are chemical agents that inactivate microorganisms, are generally broad spectrum and have many applications in clinical, domestic and industrial settings (McDonnell and Russell 1999). Table 1.6 illustrates classes of commonly used biocides, their uses and general mechanisms of action. Unlike antibiotics, the mechanisms of action of biocides are not well elucidated and biocides appear to have multiple cellular targets. The bisphenol biocide triclosan is an exception, however, and in addition to having generalised detrimental effects at higher concentrations (Regos and Hitz 1974; Gomez Escalada *et al.* 2005), acts on a specific cellular target (*enoyl–acyl carrier protein* (*ACP*) *reductase*) at lower concentrations (McMurry *et al.* 1998). The activity of biocides is influenced by numerous factors including contact period and temperature of contact, presence of interfering materials and concentration in formulation (Russell and McDonnell 2000), further complicating our understanding of their mechanisms of action.

Bacterial resistance to biocides has been recorded as far back as the early 1950s, with the identification of the tolerance of *P. aeruginosa* to the quaternary ammonium compound (QAC) cetrimide (Lowbury 1951). Subsequently *P. aeruginosa* has been recorded to have resistance to other QACs such as benzalkonium chloride (Adair *et al.* 1969; Langsrud *et al.* 2003) and didecyl dimethylammonium chloride (Langsrud *et al.* 2003), isothiazolinones (Brozel and Cloete 1994; Winder *et al.* 2000), chlorhexidine (Nakahara and Kozukue 1982), triclosan (Russell 2004), formaldehyde (Wollmann and Kaulfers 1991), zinc pyrithione (Abdel Malek *et al.* 2009) and heavy metal ions (Nakahara *et al.* 1977), amongst others. The resistance mechanisms of *P. aeruginosa* to biocides are not well defined but it seems likely that intrinsic and adaptive resistance play a large role (McDonnell and Russell 1999).

Table 1.6 Commonly used biocides: classes, mechanisms of action and uses

			General mechanisms of action	Classes	Examples	Uses
BIOCIDES	**Electrophiles**	**Oxidants**	Multiple cellular targets Oxidisation of organic material for a rapid speed of kill	Halogen releasing compounds	Chlorine compounds	Antisepsis, cleaning, disinfection
					Iodine compounds	
				Peroxycompounds	Hydrogen peroxide	Disinfection, preservation
		Electrophiles	Multiple cellular targets Inactivation of enzymes through covalent reactions with cellular nucleophiles Initiation of free radical formation	Aldehydes	Formaldehyde	Antisepsis, disinfection, preservation, sterilisation
				Formaldehyde releasing agents	Hydantoins	Antisepsis, preservation
					Oxazolidines	
				Isothialzolones	Methylisothiazolinone	Disinfection, preservation
					Chloromethylisothiazolone	
					Benzisothiazolinone	
				-	Bronopol	Preservation
				Heavy metal derivatives	Cu, Hg, Ag compounds	Antisepsis, disinfection, preservation
	Membrane active	**Protonophores**	Target mainly cell membrane Disruption of proton motive force, rendering cell membrane unable to maintain pH levels. Acidification of the cell interior and disruption of metabolism	Parabens	Methylparaben	Preservation
					Propylparaben	
					Sodium Benzoate	
				Weak Acids	Benzoic acid	Antisepsis, preservation
					Sorbic acid	
				Pyrithiones	Sodium pyrithione	Preservation
					Zinc pyrithione	
		Lytic	Target mainly cell membrane Destabilisation of membranes leading to leakage of cellular components/cell lysis	Quaternary ammonium Compounds	Cetrimide	Antisepsis, cleaning, disinfection, preservation
					Benzethonium chloride	
				Biguanides	Chlorhexidine	Antisepsis. anti-plaque agents, disinfection, preservation
				Phenols	Triclosan	Antisepsis, anti-plaque agents, preservation
					Hexachlorophene	
				Alcohols	Phenoxyphenol	Antisepsis, disinfection, preservation
					Ethyl alcohol	
					Isopropyl alcohol	

Footnotes: information from (McDonnell and Russell 1999; Chapman 2003a; Moore and Payne 2008; Abdel Malek *et al.* 2009)

1.3.2 Intrinsic resistance

P. aeruginosa demonstrates high levels of intrinsic resistance to antibiotics and biocides of different classes. Intrinsic mechanisms contribute to resistance towards antibiotics such as the β-lactams, older quinolones, chloramphenicol, tetracycline, macrolides, trimethoprim-sulfamethoxazole and rifampin (Rossolini and Mantengoli 2005) and several biocides, including QACs, triclosan, formaldehyde and chlorhexidine (Stickler 2004). This resistance is multi-factorial and largely due to the concerted action of a low overall outer membrane permeability and efflux pumps. In addition, a number of chromosomally encoded factors contribute (Hancock 1998).

Generally, Gram-negative bacteria have higher levels of intrinsic resistance than Gram-positive bacteria and this is due to the composition of the cell wall (McDonnell and Russell 1999). The Gram-negative cell wall is a complex, multi-layered structure (Figure 1.2): the outer membrane acts as a semi-permeable barrier to restrict the influx of molecules such as antimicrobials, and the periplasmic space provides a site for the metabolism of antibiotics, and possibly biocides, before they reach the cytoplasmic membrane (Hancock 1998; Russell 2002). The structure of the cell wall of *P. aeruginosa* differs slightly from other Gram-negative bacteria and is especially resistant to antimicrobial agents; it has been estimated that the outer membrane permeability of *P. aeruginosa* is over 100-fold less than that of *Escherichia coli* (Yoshimura and Nikaido 1982). This increased impermeability is partially due to a high Mg^{2+} content in the outer membrane which produces strong links between lipopolysaccharide (LPS) chains (McDonnell and Russell 1999). Additionally, the major nonspecific outer membrane porin OprF has a relatively low permeability (Nestorovich *et al.* 2006), and is an inefficient route for the uptake of antimicrobials (Hancock 1998).

Exclusion via efflux pumps, together with high membrane impermeability, prevents antimicrobial agents from reaching their cellular targets and accumulating within the cell to toxic levels. Efflux systems can be specific, facilitating the transport of one particular substrate, or general, transporting a variety of structurally unrelated compounds (Piddock 2006). Efflux pumps belonging to five superfamilies have been defined, all of which have been identified in the genome of *P. aeruginosa.* Those of the resistance-nodulation division (RND) are the most prevalent in *P. aeruginosa* with 12 RND efflux systems identified in its genome, two of which transport divalent metal cations (Lister *et al.* 2009). Ten types of RND efflux pumps found in *P. aeruginosa* (excluding the divalent metal cation transporters) and examples of their substrates are detailed in (Table 1.7). The RND family efflux systems use energy derived from the proton

motive force for the transport of compounds (Lister *et al.* 2009), and are associated with multidrug resistance, having the capacity to transport a large spectrum of substrates (Schweizer 2003).

Chromosomally encoded resistance factors also contribute to the antimicrobial resistance of *P. aeruginosa*. Similarly to several other Gram-negative species, *P. aeruginosa* possesses a chromosomally encoded AmpC β-lactamase. This β-lactamase is produced at low levels and is also induced by certain β-lactams, conferring resistance to ampicillin and most cephems (Rossolini and Mantengoli 2005; Fajardo and Martinez 2008). Resistance to formaldehyde in *P. aeruginosa* has been attributed to both outer membrane impermeability (Stickler 2004) and a chromosomally encoded formaldehyde dehydrogenase (Wollmann and Kaulfers 1991) which metabolises formaldehyde into a non-toxic metabolite (Chapman *et al.* 1998). Furthermore, triclosan resistance in *P. aeruginosa* may be compounded by the production of a chromosomally encoded, non-susceptible e*noyl*-ACP *reductase*. *P. aeruginosa* was recently demonstrated to possess two enoyl-ACP reductases, one which was triclosan-susceptible (FabI, encoded by *fabI*) and one which was refractory to triclosan inhibition (FabV, encoded by fabV) (Zhu *et al.* 2010).

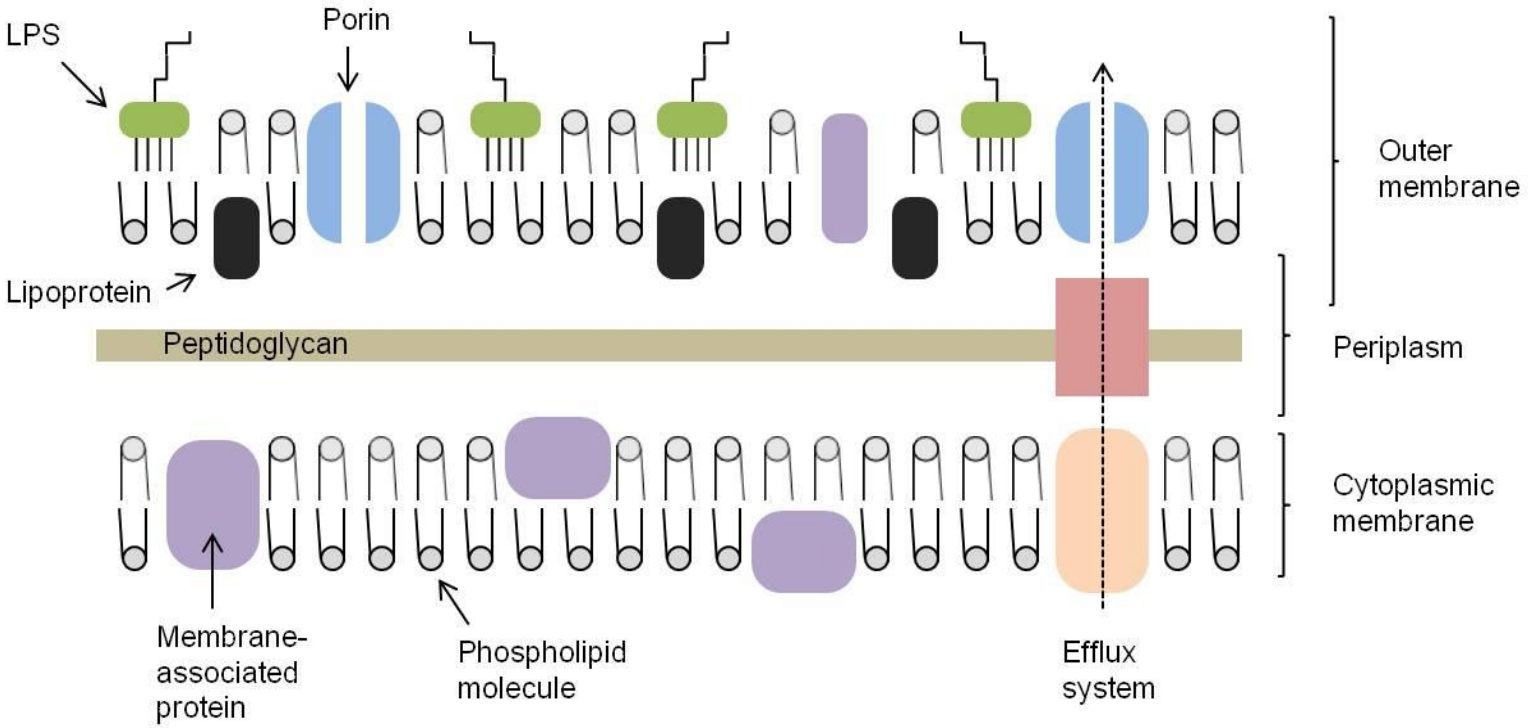


Figure 1.2 Schematic representation of the Gram-negative cell wall. The Gram-negative cell wall comprises a cytoplasmic (inner) membrane, a thin peptidoglycan layer, periplasm and an outer membrane. The cytoplasmic membrane is a phospholipid bilayer with associated proteins, whilst the outer membrane is composed of phospholipid molecules, proteins, lipoproteins, porins and lipopolysaccharide (LPS) molecules. Porins allow the entrance and egress of hydrophilic low-molecular weight substances and may be specific or non-specific. An efflux pump typical of the resistance-nodulation division (RND) family (as found in *P. aeruginosa*) is illustrated in the schematic, comprising a transporter protein which is located in the cytoplasmic membrane, a periplasmic fusion protein and an outer membrane protein. Adapted from (Hancock 1997; Hancock 1998; Piddock 2006; Madigan *et al.* 2010)

Table 1.7 Efflux systems of *Pseudomonas aeruginosa*

Efflux pump	Substrates	References
MexAB-OprM	Quinolones, fluoroquinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, β-lactams (except imipenem), trimethoprim, sulphonamides, triclosan, ethidium bromide, SDS, aromatic hydrocarbons, thiolactomycin, cerulenin, quorum sensing molecules (acylated homoserine lactones)	(Masuda *et al.* 2000) (Poole 2011) (Schweizer 2003)
MexCD-OprJ	Quinolones, fluoroquinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, penicillins (except carbenicillin and sulbenicillin), cephems (except ceftazidime), flomoxef, meropenem, S-4661, trimethoprim, crystal violet, ethidium bromide, acriflavine, SDS, aromatic hydrocarbons, triclosan	(Masuda *et al.* 2000) (Poole 2011) (Schweizer 2003)
MexEF-OprN	Fluoroquinolones, chloramphenicol, trimethoprim, tetracycline, maromatic hydrocarbons, triclosan, quorum sensing molecules (pseudomonas quinolone signal molecules)	(Köhler *et al.* 2001) (Maseda *et al.* 2000) (Poole 2011) (Schweizer 2003)
MexGHI-OpmD	Vanadium, norfloxacin, ethidium bromide, acriflavine, rhodamine 6G, quorum sensing molecules (N-acylhomoserine lactone and pseudomonas quinolone signal molecules)	(Aendekerk *et al.* 2005) (Sekiya *et al.* 2003)
MexJK*	Tetracycline, erythromycin, triclosan	(Chuanchuen *et al.* 2001)
MexMN-OprM	Chloramphenicol, thiamphenicol	(Lister *et al.* 2009)
MexPQ-PomE	Fluoroquinolones, tetracycline, chloramphenicol, erythromycin	(Lister *et al.* 2009)
MexVW*	Fluoroquinolones, tetracycline, chloramphenicol, erythromycin	(Li *et al.* 2003)
MexXY*	Aminoglycosides, quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, aminoglycosides, penicillins (except carbenicillin and sulbenicillin), cephems (except cefsulodin and ceftazidime), meropenem, S-4661	(Masuda *et al.* 2000) (Westbrock-Wadman *et al.* 1999)
TriABC-OpmH	Triclosan	(Lister *et al.* 2009)

Footnotes: *can use OprM or OpmH as the outer membrane porin component of the pump

1.3.3 Acquired resistance

Acquired resistance can arise via mutation of chromosomal genes or via acquisition of resistance genes though horizontal gene transfer (Fajardo and Martinez 2008). Mutational resistance to several antimicrobials has been reported for *P. aeruginosa* (Table 1.8). Mutations leading to the de-repression of the chromosomal β-lactamase, AmpC, can confer increased or overt resistance to β-lactams such as penicillins, cephems and monobactams. Not all of these compounds would normally induce the production of AmpC, but are sensitive to degradation by this enzyme (Rossolini and Mantengoli 2005). The loss of the outer membrane porin OprL through mutation can result in increased resistance to the carbapenem antibiotics imipenem and meropenem (Livermore 2002). In addition, the loss of OprD has been linked to resistance to the isothiazolone biocides benzisothiazolone, N-methylisothiazolone and 5-chloro-Nmethylisothiazolone (Brozel and Cloete 1994; Chapman 1998; Winder *et al.* 2000).

Mutations resulting in the upregulation of efflux systems can enhance resistance to multiple classes of antimicrobials, dependent on the substrate specificity of the pump (Table 1.8). Mutants overexpressing the efflux system MexEF-OprN also have increased resistance to imipenem, meropenem and isothiazolones due to the concomitant downregulation of OprD (Livermore 2002). Resistance to fluoroquinolones may occur via upregulation of efflux pumps or via mutations in genes encoding topoisomerases. Mutations in *gyrA* lead to the reduced susceptibility of topoisomerase II to fluoroquinolones, whilst mutations in *parC* lead to the reduced susceptibility of topoisomerase IV (Jalal and Wretlind 1998). Polymyxin resistance is believed to result from mutations which lead to changes in outer membrane structure (Rossolini and Mantengoli 2005; Poole 2011). Finally, changes in LPS structure resulting from chromosomal mutations could be responsible for reduced aminoglycoside uptake leading to increased resistance (Bryan *et al.* 1984).

Multiple acquired resistance genes encoding β-lactamases and aminoglycoside-modifiying enzymes (AMEs) have been described in *P. aeruginosa* (Table 1.8). The acquired β-lactamases belong to three different groups: (1) Narrow spectrum β-lactamases; (2) Extended spectrum β-lactamases (ESBLs); and (3) Metallo β-lactamases (MBLs). Both ESBLs and MBLs have very broad substrate profiles with MBLs being able to degrade almost all β-lactams, with the exception of monobactams (Rossolini and Mantengoli 2005). Whilst the majority of the older, narrow spectrum β-lactamases were plasmid-encoded, ESBLs and MBLs are typically encoded by genes of plasmid or transposon origin found on integrons (Poole 2011). Acquired resistance to aminoglycosides in *P. aeruginosa* has similarly been associated with AME encoding genes localised on integrons. AMEs render aminoglycosides inactive by structural modification,

typically though phosphorylation, acetylation or adenylation (Poole 2011). Details of some of the most prevalent AMEs are listed in Table 1.8. Worryingly, multiple resistance genes may be carried by integrons which can lead to multi-drug resistance, and integrons carrying genes encoding AMEs, ESBLs and MBLs have been observed (Fajardo and Martinez 2008). One further mechanism of aminoglycoside resistance is target modification via methylation of 16S rRNA through acquisition of a 16S rRNA methylase-encoding gene. This mechanism has emerged relatively recently with the recognition of the first 16S rRNA methylase, termed RmtA, in 2003 in a clinical isolate of *P. aeruginosa*. These genes are thought to be prevalent amongst Gram-negative pathogens, and are found on transposons within transferable plasmids (Strateva and Yordanov 2009).

Plasmid-mediated biocide resistance has been noted but seems to be of more significance in Gram-positive than Gram-negative bacteria, where intrinsic mechanisms play the major role (Russell 1997). In *P. aeruginosa* there have been few reports of plasmid-encoded biocide resistance aside from resistance to hexachlorophene (Sutton and Jacoby 1978), mercury and organomercurial compounds (Clark *et al.* 1977). The significance of increased resistance to hexachlorophene is unknown, however, as this compound has a much greater effect upon Gram-positive bacteria than Gram-negative bacteria (McDonnell and Russell 1999).

Table 1.8 Acquired resistance mechanisms of *Pseudomonas aeruginosa*

Resistance mechanism	Affected antimicrobial agents
Mutation of chromosomal genes	
De-repression of AmpC	Penicillins, cephems, monobactams
Loss of OprD	Imipenem, meropenem, isothiazolones
Upregulation of efflux systems	
- MexAB-OprM	All β-lactams except imipenem, fluoroquinolones
- MexCD-OprJ	Some β-lactams (cefoperazone, cefpirome, cefepime, meropenem), fluoroquinolone
- MexEF-OprN (concomitant OprD downregulation)	Fluoroquinolones (imipenem, meropenem, isothiazolones)
- MexXY-OprM	Some β-lactams (cefoperazone, cefpirome, cefepime, meropenem), fluoroquinolones, aminoglycosides
Mutations in *gyrA* and/or *parC*	Fluoroquinolones
Mutations leading to changes in outer membrane structure	Polymyxins, aminoglycosides
Acquisition of resistance genes via horizontal gene transfer	
β-lactamases	
- Narrow spectrum Molecular class A (e.g. PSE-1, PSE-4, TEM-1) Molecular class D (e.g. OXA-3)	Penicillins, cefoperazone
- Extended spectrum Molecular class A (e.g. PER-1, VEB-1, GES-1, GES-2, TEM-42, SHV-5) Molecular class D (e.g. OXA-11, OXA-14, OXA-18, OXA-28)	Penicillins, cephems, monobactams
- Metallo-enzymes Molecular class B (e.g. IMP-, VIM-, SPM-, GIM-type)	All β-lactams except monobactams
Aminoglycoside-modifying enzymes	Aminoglycosides
- AAC(3)-I	Gentamicin
- AAC(3)-II	Gentamicin, tobramycin
- AAC(6')-I	Tobramycin, netilmicin, amikacin
- AAC(6')-II	Gentamicin, tobramycin, netilmicin
- ANT(2')-I	Gentamicin, tobramycin
16S rRNA methylases (e.g. RmtA)	Aminoglycosides - amikacin, tobramycin, isepamicin, kanamycin, arbekacin, gentamicin
Plasmids pMG1 and pMG2	Hexachlorophene
Plasmids pMG1, pMG2, R26, R933, R93-1, pVS1 FP2, R38, R3108 and pVS2	Mercury, organomercurial compounds

Footnotes: information from (Clark *et al.* 1977; Sutton and Jacoby 1978; Livermore 2002; Rossolini and Mantengoli 2005; Strateva and Yordanov 2009)

1.3.4 Adaptive resistance

Adaptive resistance is a phenomenon that is distinct from both intrinsic and acquired resistance, and is also less well understood. Characterised by resistant phenotypes which are transient, rather than irreversible, adaptive resistance is triggered by exposure to a particular environmental stimulus, and may lead to increased resistance to one or more antimicrobial compounds. Generally, as the stimulus subsides, so does the resistance phenotype, although this is not always the case. Initially it was thought that exposure to sub-inhibitory concentrations of an antimicrobial acted as the stimulus for adaptive resistance. Now it is known that in addition to this, a variety of other environmental cues are involved. These can include pH, anaerobiosis and nutrient levels, as well as social behaviour such as biofilm formation (see section 1.3.5) and swarming motility. The pathways involved in adaptive resistance are complex and only beginning to be understood (Fernández *et al.* 2011). Adaptive resistance to both antibiotics and biocides has been described for *P. aeruginosa* and will be discussed.

Environmental conditions such as anaerobiosis (Haussler *et al.* 1999), Mg^{2+} limitation (McPhee *et al.* 2006), carbon source availability (Conrad *et al.* 1979) and pH (Xiong *et al.* 1996) have been shown to trigger an adaptive resistance response in *P. aeruginosa*, increasing resistance to antibiotics such as aminoglycosides, polymyxins and amikacin. In addition, exposure to sub-inhibitory concentrations of some antibiotics has also led to adaptive resistance through a number of pathways. For example, exposure to aminoglycosides has been shown not only to induce biofilm formation (Karatan and Watnick 2009) and upregulate MexXY (Hocquet *et al.* 2003), but also to upregulate genes involved in anaerobic metabolism (Karlowsky *et al.* 1997). This may confer protection against antibiotic killing as the aerobic respiratory pathway is required for cytoplasmic accumulation of aminoglycosides (Fernández *et al.* 2011). Exposure to sub-inhibitory concentrations of β-lactams causes the induction of the chromosomal AmpC β-lactamase and has also been found to upregulate genes involved in multidrug efflux and antibiotic resistance (Blázquez *et al.* 2006). The increased expression of the MexAB-OprM efflux system has been observed on exposure of *P. aeruginosa* to quinolone antibiotics, which would contribute to adaptive resistance through active efflux of antibiotics (Fernández *et al.* 2011). Quinolone exposure can also induce the SOS response (Brazas and Hancock 2005) which can combat DNA damage caused by the antibiotics and also lead to a transient increase in mutation frequency. This transitory hypermutation state could lead to the permanent acquisition of resistance to one or more antibiotics, a phenomenon known as adaptive mutation (Fernández *et al.* 2011). Furthermore, sub-inhibitory concentrations of polymyxins have been demonstrated

to upregulate expression of the LPS modification gene operon. The modification that is induced by this pathway decreases the net negative charge of the LPS rendering the outer membrane less able to bind positively charged antimicrobials (Fernández *et al.* 2011).

The mechanisms of adaptive resistance to biocides are poorly understood and mostly believed to be non-specific. Multiple mechanisms of adaptive resistance have been observed during the sub-culturing of *P. aeruginosa* in media containing increasing concentrations of biocide, including: (i) outer membrane alterations on exposure to QACs (Loughlin *et al.* 2002) and zinc pyrithione (Abdel Malek *et al.* 2009); (ii) changes in the fatty acid profiles of the outer membrane on exposure to QACs and amphoteric surfactants (Jones *et al.* 1988; Loughlin *et al.* 2002), and; (iii) increased active efflux on exposure to zinc pyrithione (Abdel Malek *et al.* 2009). Worryingly, adaptive resistance to biocides in *P. aeruginosa* has been associated with increased resistance to other classes of biocides and antibiotics (Adair *et al.* 1969; Jones *et al.* 1988; Loughlin *et al.* 2002; Langsrud *et al.* 2003; Abdel Malek *et al.* 2009; Abdel Malek and Badran 2010). Cross resistance can occur if antimicrobials use the same pathway to reach their target or have a similar mechanism of action (Chapman 2003b). The resistance mechanisms triggered by biocide exposure are non-specific and it is likely that overall changes in membrane permeability or increased activity of efflux pumps (especially those with broad specificity) could increase resistance to multiple antimicrobial agents simultaneously. It is therefore a possibility that the widespread and indiscriminatory use of biocides, sometimes at sub-inhibitory concentrations, is contributing to the increasing levels of antimicrobial resistance (Fernández *et al.* 2011).

1.3.5 Biofilm formation

Biofilms are surface-attached bacterial communities surrounded by an exopolysaccharide matrix (Drenkard 2003). The occurrence of biofilms in natural, industrial and medical habitats is common and it has been proposed that biofilms are the preferred mode of growth of bacterial cells (Costerton 1999; Stickler 2004). Cells within biofilms exhibit growth characteristics different to those of planktonic cells and in particular are more resistant to antibiotics and biocides; as mentioned previously biofilm formation is recognised as a form of adaptive resistance (Fernández *et al.* 2011). This poses a problem to the effective treatment of infection, as biofilms may form on prosthetic devices (Stickler 2004) as well as living tissues, such as within the cystic fibrosis lung (Davies and Bilton 2009). In industry biofilm formation on the surfaces of equipment and machinery can lead to product contamination and process inefficiency (Stickler 2004).

The resistance of *P. aeruginosa* biofilms to antibiotics has been observed, sometimes to extreme levels. For example, Nickel *et al.* (Nickel *et al.* 1985) demonstrated that biofilms of *P. aeruginosa* could be up to 1000-fold more resistant to tobramycin than planktonic cells. Biofilm-mediated resistance to biocides has also been noted: biofilms of *P. aeruginosa* grown on polycarbonate surfaces were reported by Brown *et al.* (Brown *et al.* 1995) to have increased resistance to povidone iodine. Additionally, Cochran *et al.* (Cochran *et al.* 2000) found that biofilms of *P. aeruginosa* attached to alginate beads exhibited enhanced resistance to hydrogen peroxide and monochloramine in comparison to planktonic cells. Biofilm-mediated antimicrobial resistance is complex, multifactorial and not completely understood. Several mechanisms for resistance have been postulated (Drenkard 2003):

- Restricted antimicrobial penetration: the exopolysaccharide matrix may delay the penetration of some antimicrobials such as aminoglycosides into *P. aeruginosa* biofilms (Shigeta *et al.* 1997).

- Cells within the biofilm have limited access to oxygen and nutrient and exhibit decreased metabolic activity. This may increase antimicrobial tolerance as antimicrobials are more effective against metabolically active cells (Drenkard 2003). Growth under anaerobic conditions in *P. aeruginosa* biofilms has led to increased resistance to antibiotics such as tobramycin, ciprofloxacin, carbenicillin, ceftazidime, chloramphenicol and tetracycline (Borriello *et al.* 2004).

- Biofilm-specific phenotype: biofilm bacteria and planktonic have different gene and protein expression profiles. The physiological, metabolic and phenotypic changes occurring in cells in the developing biofilm may be important in the activation of resistance mechanisms (Drenkard 2003).

- Persister cells and highly resistant phenotypic variants: these two types of antimicrobial resistant variants exist as subpopulations within the biofilm and promote biofilm survival on repeated exposure to antimicrobial agents (Drenkard 2003).

- Activation of a generalised protective stress response and possible upregulation of multidrug efflux pumps (Drenkard 2003).

- Interaction of the biofilm with antimicrobials: some strains of *P. aeruginosa* synthesise glucans which can be incorporated into the biofilm and bind aminoglycosides (Poole 2011).

Regulation of biofilm formation is complex and mediated by numerous environmental cues including mechanical, metabolic and nutritional signals, the availability of inorganic ions, osmolarity, host-derived factors, antimicrobials (such as tobramycin which induces biofilm formation in *P. aeruginosa*) and quorum sensing (QS) signals (Karatan and Watnick 2009). QS circuits are very important in virulence and biofilm formation in *P. aeruginosa*; it has been observed that mutants with defective QS components could not form biofilms of the regular structure and had increased susceptibility to sodium dodecyl sulphate (Davies *et al.* 1998; Stickler 2004). Therefore, an attractive method for the control of *P. aeruginosa* biofilms is the disruption of QS signals. Promisingly, potential targets for the disruption of QS in *P. aeruginosa* have been identified (del Pozo and Patel 2007), for example small molecule inhibitors of the quorum sensing receptors LasR and RhlR have been described by O'Loughlin *et al.* (O'Loughlin *et al.* 2013).

1.4 Project aims

The overall aim of the PhD was to investigate *P. aeruginosa* as an important and common contaminant of home and personal care products. The different areas that were covered are summarised in Figure 1.3. This PhD was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and being a BBSRC-CASE studentship, was also sponsored by Unilever Research and Development UK. Several of the questions explored during this study were guided by the interests of Unilever, with the goal of informing industrial practices.

Specific project aims were as follows:

1. **Evaluate culture-dependent methods for the detection of *P. aeruginosa* using a diverse strain panel (Chapter 3)**. Five agars for the detection of *P. aeruginosa* were screened for sensitivity and specificity using a large strain panel originating from clinical, environmental and industrial sources. In addition, the utility of acetamide-based selective media as enrichment media for *P. aeruginosa* was assessed. A pre-requisite to these investigations was the establishment of a collection of *P. aeruginosa* and non-*P. aeruginosa* strains from industrial sources. As culture-dependent methods are a mainstay of contaminant detection in industry, this chapter informed Unilever of current best practices.

2. **The design and evaluation of novel PCRs for the detection of *P. aeruginosa* and investigation of the bacterial diversity associated with home and personal care products (Chapter 4).** Two novel species-specific PCRs were designed to detect *P. aeruginosa* and evaluated for sensitivity and specificity using a large, genetically diverse strain panel. The applicability of culture-independent techniques to contaminant detection from industrial products was assessed. Subsequent investigations were performed to examine the bacterial diversity encountered in home and personal care products.

3. **The examination of *P. aeruginosa* strain diversity in industry and its relation to the wider population biology of the species (Chapter 5)**. A large collection of *P. aeruginosa* strains isolated from industrial products was subjected to multiple strain-typing techniques to determine any associations between genotype and product type, time or location of isolation. Relation to a broad database of other *P. aeruginosa* strains

from clinical and environmental habitats facilitated investigations into population structure.

4. **Characterisation of a *P. aeruginosa* strain panel originating from industrial sources (Chapter 6).** The preservative susceptibility of a panel of *P. aeruginosa* strains from clinical, environmental and industrial sources was determined, in addition to growth and motility characteristics. Putative differences between isolation source and phenotypic profiles were examined. In addition, the development of adaptive resistance to a preservative combination was examined using both planktonic growth and biofilm models.

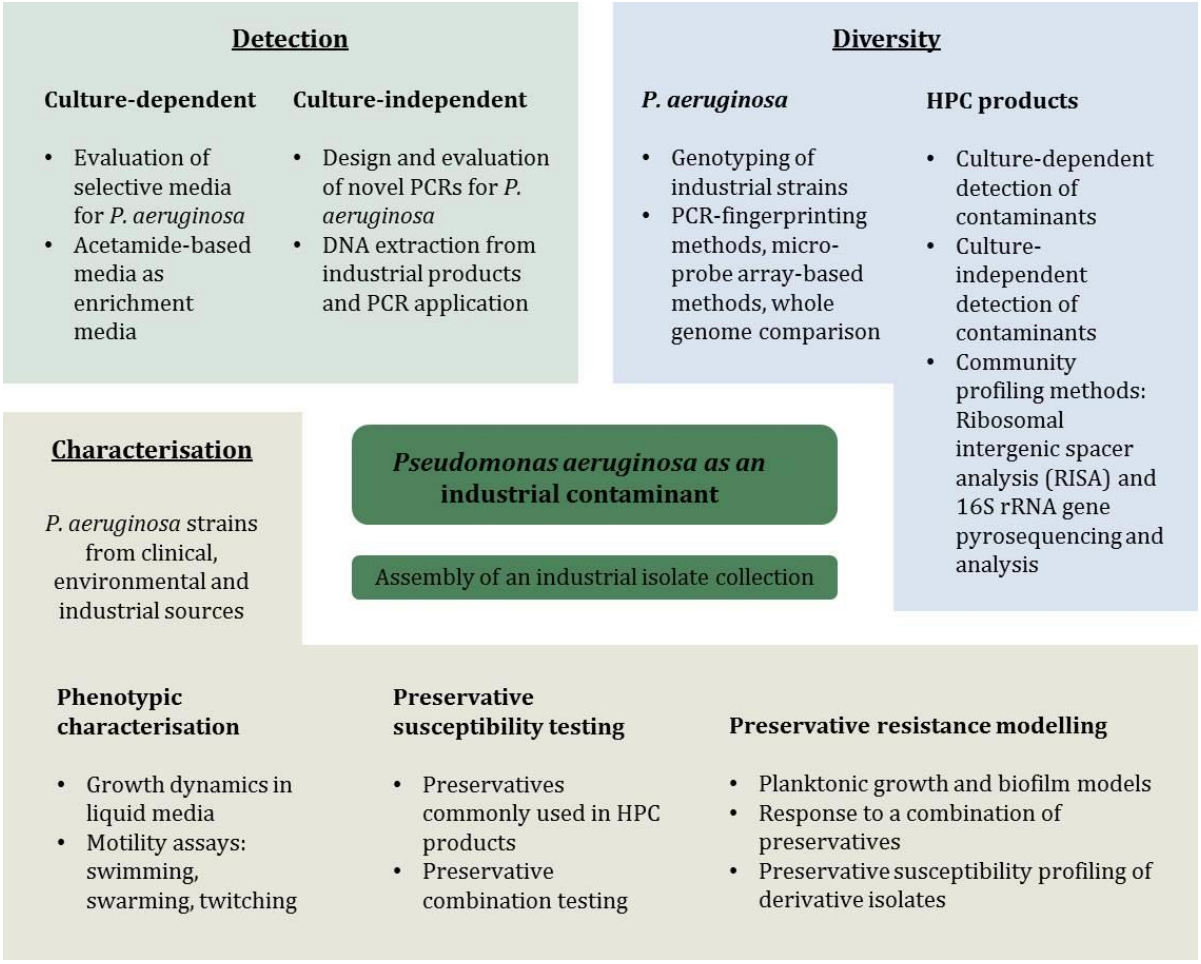


Figure 1.3 Key focus areas of the PhD. To investigate *P. aeruginosa* as an industrial contaminant, four main areas were considered: (1) detection (Chapters 3 and 4); (2) diversity (Chapters 4 and 5); (3) strain characterisation (Chapter 6), and; (4) modelling of adaptive resistance to preservatives (Chapter 6). All of the studies were underpinned by the assembly of a large industrial isolate collection (Chapter 3). HPC, home and personal care.

2. Methods & materials

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich Company Ltd. (Dorest, UK) and Fisher Scientific (Loughborough, UK), unless otherwise stated. Aqueous solutions of chemicals were prepared in double-deionised water and sterilised either by autoclaving at 121°C for 15 minutes, or filtration using Minisart® syringe filters (pore size 0.2 µm; Sartorius Stedim Biotech, UK) for small volumes.

2.2. Media

All media were prepared with double-deionised water and sterilised by autoclaving at 121°C for 15 minutes. Where applicable the media were prepared according to the manufacturer's instructions. Tryptone Soya Agar (TSA; Oxoid Ltd., Basingstoke, UK) and Tryptone Soya Broth (TSB; Oxoid Ltd.) were routinely used growth media for bacterial isolates. Selective media for the growth of *P. aeruginosa* included Pseudomonas CN selective agar (PCN; Oxoid Ltd.), Pseudomonas isolation agar (PIA; Sigma Aldrich Complany Ltd, Dorset, UK), chromID® *P. aeruginosa* (chromID Pa; bioMérieux UK Ltd, Basingstoke, UK; supplied as pre-poured plates), Z-agar (Szita *et al.* 1998), and modified Z-agar (mZ-agar) which had the same formula as Z-agar but with the addition of 0.01% (w/v) Bacto™ Casamino acids (BD UK Ltd., Oxford, UK) and 0.01% (w/v) yeast extract (Oxoid Ltd.). Other media used included non-cation adjusted Mueller-Hinton (MH) broth (Oxoid Ltd.) for growth curve experiments, and modified Z-broth for enrichment assays. The formulation of the Z-broth was modified from the original (Szita *et al.* 1998) and was as follows: acetamide 4 g/L (concentration adjusted for enrichment assays from 1 – 50 g/L), dipotassium hydrogen phosphate (5 g/L), potassium dihydrogen phosphate (1 g/L), potassium sulphate (2 g/L) and magnesium sulphate heptahydrate (0.05 g/L)

2.3. Preparation of antimicrobial agents

2.3.1. Preservatives and preservative enhancing agents

Storage of all preservatives and preservative enhancing agents was at room temperature with the exception of phenyl-arginine-beta-naphthylamide (PAβN; MC-207,110; Sigma-Aldrich) which was stored at -20°C. Stock solutions of the preservatives: CITMIT (Kathon CG [CIT and MIT blend]; Dow Europe GmbH, Switzerland); BIT (Koralone B-120; Dow Europe GmbH); MIT (Neolone M10; Dow Europe GmbH); PHE (Phenoxetol; Clariant Produkte GmbH, Germany) and

CHX (chlorhexidine digluconate; Sigma-Aldrich Company Ltd.); and preservative boosters PAβN, CX-3 (Merck KGaA, Germany), CX-7 (Merck KGaA) and Velsan SC (VSC; Clariant Produkte GmbH), were prepared in polished water. Benzoic acid (BA; Sigma-Aldrich Company Ltd.) was dissolved in an aqueous solution of 20% dimethylsulphoxide (DMSO). Further dilutions for minimum inhibitory concentration and growth assays were prepared from these solutions, detailed in the relevant sections. Stocks for viscous agents (Koralone B-120, CX-3, CX-7 and VSC) were prepared by weight, rather than volume, as they were difficult to accurately pipette; in these stocks the weight of the equivalent volume of water was added, and calculations performed in a % (v/v) manner. Stocks of all agents were prepared on the day of use and stored for no longer than 2 days at 4°C (except PAβN which was kept at -20°C) before discarding and making fresh.

2.4. Bacterial strains and routine growth conditions

2.4.1. Strain panel

Bacterial strains were drawn from the Mahenthiralingam group strain collection at Cardiff University and copies of the international *Pseudomonas aeruginosa* reference panel (De Soyza *et al.* 2013) and EuroCareCF strain panel (European Coordination Action for Research in Cystic Fibrosis; EC FP6 project no. LSHM-CT-2005-018932) that were held at Cardiff University. Challenge test strains and contamination isolates were also obtained from freezer stocks held at Unilever Research and Development Port Sunlight (URDPS). Additional industrial strains were isolated from contaminated home and personal care products archived at URDPS (described in section 2.8.1). Species and strains used in experiments are detailed in the relevant sections.

2.4.2. Growth and storage of bacterial isolates

Bacterial isolates were routinely grown on TSA or in TSB overnight (16-18 hours) at either 30°C or 37°C, stated in the relevant sections. Overnight cultures were prepared by inoculating 3 ml of TSB with fresh (<72 hours) growth material collected using a sterile pipette tip from a pure streak plate, and incubated on an orbital shaker (150 rpm). Freezer stocks of individual isolates were prepared in TSB containing 8% (v/v) DMSO and stored at -80°C.

2.5. Growth curve analysis in liquid media

2.5.1. Growth in the Bioscreen C

To examine growth dynamics of *P. aeruginosa* strains a Bioscreen C instrument (Labsystems, Finland) was used. Each Bioscreen C microplate well contained 200 µl liquid medium

inoculated with approximately 10^5 cfu/ml from an overnight culture. The liquid medium used and whether the medium was supplemented with preservatives was experiment-dependent, and will be described in the relevant methods sections. Growth was monitored for 48 hours at 37°C; turbidity measurements were taken at 15 minute intervals using a wide band filter (450-580 nm) after shaking the microplates for 10 seconds at an intermediate intensity. Each experiment was performed twice with different starting overnight cultures and contained 4 technical replicates; data was pooled from these cultures for analysis as stated below.

2.5.2. Data handling and estimation of growth parameters

The data from the Bioscreen C were exported into Microsoft Excel and the mean value of the broth-only control wells subtracted from all test samples. Subsequently, the optical density (OD) data for each sample were averaged and logarithmically transformed. To quickly visually assess the data, scatterplots were constructed in Microsoft Excel. The gcFit function of the grofit statistical package (Matthias *et al.* 2010) for use in R statistical software (R-Core-Team 2013) was used to estimate the following growth parameters for logarithmically transformed data, i) length of lag phase, ii) maximum growth rate, and iii) maximum culture density reached. Strains producing growth curves which could not be modelled accurately by grofit (discordance between model and model-free-spline fits) were excluded.

2.5.3. Comparison of growth parameters

To examine the distribution of the growth parameter data, boxplots were generated using BoxPlotR (Michaela *et al.* 2014). The growth parameter data was analysed with non-parametric statistical methods. Using R statistical software, a Kruskal-Wallis test and post-hoc Wilcoxon tests with Benjamini-Hochberg correction were used to determine if the medians of the strain groups from different isolation sources were significantly different at the p=0.05 level.

2.6. DNA extraction

2.6.1. Rapid DNA extraction using Chelex®100 resin

Material from a single colony was transferred into 50 µl 5% Chelex® 100 resin solution (Bio-rad, Hertfordshire, UK; autoclaved prior to use) using a sterile pipette tip. The sample was heated to 98ºC in a c1000™ Thermal Cycler (Bio-Rad) for 5 minutes, then placed at 4°C for 5 minutes. This process was repeated before centrifuging the sample at 800 *g* for 1 minute to sediment the Chelex® 100 resin and cellular debris. Crude DNA from the supernatant was used

in PCR reactions. Storage of the DNA was at 4°C and DNA was used on the same day as the Chelex® preparation was performed.

2.6.2. Promega Wizard® Genomic DNA purification kit

DNA extraction using the Wizard DNA purification kit (Promega) was performed according to the manufacturer's instructions (extraction from Gram-negative bacteria) with the following modifications: 5 ml of an overnight bacterial culture was harvested by centrifugation, the supernatant discarded, and 600 µl nuclei lysis solution added to the cell pellet. The remainder of the protocol was followed exactly, with the exception that the harvested DNA was re-suspended in 100 µl low EDTA TE buffer (10mM Tris-HCI, 0.01mM EDTA, pH 8.0) rather than the DNA re-suspension buffer provided with the kit. Storage of the DNA was at -20°C

2.6.3. Automated DNA extraction using the Maxwell® 16 system

Genomic DNA extraction from pure bacterial cultures and total DNA from home and personal care products was achieved using the Maxwell® 16 instrument (Promega) and the Maxwell® 16 Tissue DNA purification kit, according to the manufacturer's instructions. For extraction from pure cultures a 3 ml overnight culture was centrifuged (2054*g* for 10 minutes) to harvest the cells, the pellet resuspended in 300 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8) (Sambrook *et al.* 1989) and added into the DNA purification kit cartridges. For extraction from home and personal care products, 0.3 g of each product was added directly into the DNA purification kit cartridges. RNase A was added to eluted DNA to a final concentration of 0.5 µg/ml and the solution incubated at 37°C for 1 hour.

2.6.4. Quantitation and quality assessment of extracted DNA

Genomic DNA was analysed by gel electrophoresis using 1% (w/v) agarose (molecular grade agarose; Severn Biotech Ltd.) gels stained with SafeView stain (NBS Biologicals Ltd., Cambridgeshire, UK; 10 µl SafeView per 100 ml of agarose gel) and run at 80 V for approximately 1.5 hours. Quantitation of genomic DNA was performed using the Quantifluor® dsDNA system (Promega, Wisconsin, USA) kit and the Qubit™ fluorometer (Invitrogen, Massachusetts, USA), following the manufacturer's instructions. DNA quality was assessed using the absorbance ratio at 260 and 280 nm ($A_{260\ nm}/A_{280\ nm}$) using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA); ratios of approximately 1.8 were taken as an indication of good quality DNA. Any necessary dilutions of DNA were performed using sterile nuclease free water (Severn Biotech Ltd.).

2.7. Molecular taxonomy: species-level identification of bacterial isolates and analysis of gene sequences

2.7.1. Marker gene amplification, sequencing and species identification

2.7.1.1. PCR reaction volumes and conditions

Amplification of fragments of the 16S rRNA, *rpoD*, *recA* and *gyrB* genes was achieved using the primers described in Table 2.1. PCR reactions had a total volume of 25 µl containing 1X Coralload buffer, 1X Q-solution, 200 µm deoxynucleoside triphosphates (dNTPs), 1.6 µm each primer, 1 U *Taq* polymerase and 2 µl DNA template (approximately 20 ng). All reagents were supplied by Qiagen (Limburg, Netherlands). PCR cycles were performed on a c1000™ Thermal Cycler (Bio-Rad) and are shown in Table 2.2. Gel electrophoresis was performed on 1.5% (w/v) agarose gels stained with SafeView (10 µl SafeView per 100 ml of agarose gel). PCR products were visualised with a UV transilluminator and gel images captured using GeneSnap Software. PCR products were purified using the QIAquick PCR Purification kit (Qiagen) before submission for Sanger sequencing.

Table 2.1 Primers used to amplify marker genes for species identification

Target gene	Primer pair	Primer sequence 5'>3'	Annealing temp (°C)	Target group	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	52	Bacteria (Universal)	(Lane *et al.* 1985)
	1492R	GGTTACCTTGTTACGACTT			
rpoD	PsEG30F	ATYGAAATCGCCAARCG	54	*Pseudomonas*	(Mulet *et al.* 2009)
	PsEG790R	CGGTTGATKTCCTTGA			
recA	F	AGGACGATTCATGGAAGAWAGC	58	*Burkholderia*	(Spilker *et al.* 2009)
	R	GACGCACYGAYGMRTAGAACTT			
gyrB	F	ACCGGTCTGCAYCACCTCGT	60	*Burkholderia*	(Spilker *et al.* 2009)
	R	YTCGTTGWARCTGTCGTTCCACTGC			

Table 2.2 PCR cycles for the amplification of marker genes for species identification

PCR step		16S rRNA gene			*rpoD*, *recA*, and *gyrB* genes		
		Temp (°C)	Time (mins)	No cycles	Temp (°C)	Time (mins)	No cycles
1	Initial DNA denaturation	95	2	1	94	5	1
2	DNA denaturation	94	0.5	35	94	1	30
3	Primer annealing	52	0.5		[a]	1	
4	Primer extension	72	1.5		72	1.5	
5	Final extension	72	5	1	72	10	1
6	Indefinite hold	10	-	-	10	-	-

[a] Annealing temperatures vary for *rpoD*, *recA* and *gyrB* primer sets (see Table 2.1)

2.7.1.2. Gene sequencing and data processing

Purified PCR products were submitted to the MWG Eurofins DNA Sanger sequencing service following the sample submission guidelines for 'MWG Eurofins Value Read Service in Tubes'. The forward and reverse primers for each gene fragment were sent together with the PCR products in order to obtain sequences for both the forward and reverse strands. Using BioEdit Sequence Alignment Editor (Hall 1999), consensus gene sequences for individual isolates were built from the forward and reverse sequences generated by Sanger Sequencing. The 16S rRNA gene consensus sequences were compared to sequences of cultivable bacteria in the Ribosomal Database Project (RDP) II (Cole *et al.* 2009). The closest sequences matches from the RDP II database were used to assign isolates to a particular species. Database searches conducted at the NCBI taxonomy database (Sayers *et al.* 2009), the *Pseudomonas* genome database (Winsor *et al.* 2009) and the *Burkholderia* genome database (Winsor *et al.* 2008) using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.* 1990) were used to infer species identities from the *rpoD*, *recA* and *gyrB* consensus gene sequences.

2.7.2. Sequence alignment and phylogenetic tree construction

Gene sequences were imported into MEGA 6 (Tamura *et al.* 2013), trimmed, and aligned using ClustalW2 (Larkin *et al.* 2007). A neighbour-joining tree based on 1000 bootstrap replicates was constructed in MEGA 6 using the Jukes-Cantor model. Bootstrap values were calculated as percentages of 1000 replications. Trees were drawn to scale and evolutionary distances computed using the Jukes-Cantor method, with units being the number of base substitutions per site.

2.8. Culture-dependent detection methods

2.8.1. Isolation of industrial isolates from URDPS freezer archives

URDPS holds a large collection of microbial isolates from previous contamination incidents stored on beads within cryogenic vials at -80°C. To obtain isolates for the RW collection at Cardiff, one cryogenic bead from a freezer vial was streaked over the surface of a TSA plate and allowed to grow for a maximum of 48 hours at 30°C. Growth from pure streak plates was stored on transport swabs (M40 Transystem charcoal swabs; Copan Diagnostics Inc., CA, USA) which were sent back to Cardiff University. Once at Cardiff, the swabs were re-streaked onto TSA to ensure purity, and freezer stocks prepared in TSB containing 8% (v/v) DMSO. The complete industrial isolate panel is shown in Table 2.3.

2.8.2. Isolation of cultivable bacteria from home and personal care samples

A range of contaminated home and personal care products sourced from the contaminated product library at URDPS were sampled for bacterial growth (Table 2.4). To isolate cultivable bacteria and determine total viable counts, tenfold serial dilutions (10^0 to 10^{-7}) of products were performed in TSB and 100 µl spread onto TSA plates. After incubation at 30°C for a maximum of 5 days the numbers and types of different colony morphologies were recorded, streaked to purify on fresh TSA plates and freezer stocks prepared in TSB containing 8% DMSO. Total viable counts in cfu/ml were calculated from the number of colonies recorded. Serial dilutions of viscous products (dish wash liquids, liquid abrasive cleaners, fabric washes and personal care products) were prepared by weight, rather than volume, as they were difficult to accurately pipette; in these dilutions the weight of the equivalent volume of water was added, for example 1 g product plus 9 ml diluent for a tenfold dilution. If required, a neutralisation step was performed to encourage more bacterial growth; tenfold serial dilutions were performed as above except using an aqueous solution containing 2% (w/v) peptone and 1% (v/v) Tween 80 as the diluent. Each dilution was exposed to the neutralising agent for no longer than 10 minutes before spreading on TSA plates. The complete industrial isolate panel is shown in Table 2.3.

Table 2.3 Industrial isolate panel

RW#	Genus/Species ID	Comment
109	*Pseudomonas aeruginosa*	Challenge test strain used in preservative efficacy testing
110	*Pseudomonas aeruginosa*	Challenge test strain used in preservative efficacy testing
111	*Pseudomonas pseudoalcaligenes*	Challenge test strain used in preservative efficacy testing
112	*Pseudomonas putida*	Challenge test strain used in preservative efficacy testing
113	*Pseudomonas aeruginosa*	SC; 2011
114	*Pseudomonas aeruginosa*	SC; 2011
115	*Pseudomonas aeruginosa*	SC; 2011
116	*Pseudomonas aeruginosa*	SC; 2011
117	*Pseudomonas aeruginosa*	DWL; 2010; Indonesia
118	*Pseudomonas aeruginosa*	DWL; 2010; Indonesia
119	*Pseudomonas aeruginosa*	DWL; 2010; Italy
120	*Pseudomonas aeruginosa*	DWL; 2010; Italy
121	*Pseudomonas aeruginosa*	DWL; 2010; Italy
122	*Pseudomonas aeruginosa*	DWL; 2009; Italy;
123	*Pseudomonas aeruginosa*	DWL; 2011; Malaysia
124	*Pseudomonas aeruginosa*	DWL; 2009; Italy
125	*Pseudomonas aeruginosa*	DWL; 2009; Italy
126	*Pseudomonas aeruginosa*	DWL; 2009; Italy
127	*Pseudomonas aeruginosa*	DWL; 2010; Indonesia
128	*Pseudomonas aeruginosa*	DWL; 2010; Italy
129	*Pseudomonas aeruginosa*	DWL; 2010; Italy
130	*Pseudomonas aeruginosa*	DWL; 2010; Italy
131	*Pseudomonas aeruginosa*	DWL; 2010; Italy
132	*Pseudomonas aeruginosa*	DWL; 2010; Italy
133	*Pseudomonas aeruginosa*	DWL; 2010; Thailand
134	*Pseudomonas aeruginosa*	DWL; 2010; Thailand
135	*Pseudomonas aeruginosa*	DWL; 2010; Indonesia
136	*Pseudomonas aeruginosa*	DWL; 2010; Indonesia
137	*Pseudomonas aeruginosa*	DWL; 2010; Italy
138	*Pseudomonas aeruginosa*	LAC; 2001
139	*Pseudomonas aeruginosa*	LAC; 2001
140	*Pseudomonas aeruginosa*	DWL
141	*Pseudomonas spp.*	DWL
142	*Pseudomonas spp.*	DWL
143	*Pseudomonas aeruginosa*	DWL
144	*Pseudomonas aeruginosa*	DWL
145	*Pseudomonas aeruginosa*	DWL; 2010
146	*Pseudomonas aeruginosa*	DWL; 2004; Italy
147	*Pseudomonas aeruginosa*	PC
148	*Pseudomonas aeruginosa*	PC
149	*Pseudomonas aeruginosa*	PC
150	*Pseudomonas aeruginosa*	PC
151	*Pseudomonas aeruginosa*	strain ATCC 9027 used in period after opening studies
152	*Burkholderia vietnamiensis*	PC; 2002
153	*Pseudomonas putida*	PC
154	*Pseudomonas putida*	PC; 2003
155	*Pseudomonas spp.*	PC
156	*Burkholderia cenocepacia*	PC
157	*Pseudomonas putida*	PC
158	*Pseudomonas putida*	DWL
159	*Pseudomonas putida*	DWL
160	*Ochrobactrum anthropi*	RM
161	*Pseudomonas putida*	RM; 2005
162	*Bacillus spp.*	RM; 2009
163	*Pseudomonas spp.*	RM; 2004

164	*Pseudomonas spp.*	RM
165	*Pseudomonas spp.*	Misc
166	*Pantoea agglomerans*	Misc; 2002
167	*Pseudomonas spp.*	Misc
168.1	*Serratia marcescens*	FWS
168.2	*Pseudomonas aeruginosa*	FWS
169	*Pseudomonas spp.*	FWS
170	*Pseudomonas putida*	SC
171	*Halomonas spp.*	SC
172	*Pseudomonas aeruginosa*	DWL; 2009
173	*Pseudomonas aeruginosa*	DWL; 2011
174	*Pseudomonas aeruginosa*	DWL; 2010
175	*Pseudomonas aeruginosa*	DWL; 2010
176	*Pseudomonas aeruginosa*	DWL; 2010
177	*Pseudomonas aeruginosa*	DWL; 2010
178	*Pseudomonas aeruginosa*	DWL; 2010
179	*Pseudomonas aeruginosa*	DWL; 2010
180	*Pseudomonas aeruginosa*	DWL
181	*Pseudomonas aeruginosa*	DWL
182	*Pseudomonas aeruginosa*	DWL
183	*Pseudomonas spp.*	DWL
184	*Pseudomonas aeruginosa*	DWL
185	*Pseudomonas aeruginosa*	SC
186	*Burkholderia spp.*	SC
187	*Pseudomonas aeruginosa*	PC
188	*Pseudomonas aeruginosa*	PC
189	*Pseudomonas aeruginosa*	PC
190	*Pseudomonas aeruginosa*	SC
191	*Pseudomonas aeruginosa*	SC
192	*Pseudomonas aeruginosa*	SC
193	*Pseudomonas aeruginosa*	PC; 2003
194	*Pseudomonas aeruginosa*	DWL; 2011; Trinidad and Tobago
195	*Pseudomonas aeruginosa*	DWL; 2006
196	*Pseudomonas aeruginosa*	ATCC 13388; reference strain used in industrial testing (ISO 846C); origin unknown
197	*Pseudomonas aeruginosa*	ATCC 10145; reference strain used in industrial testing,
198	*Pseudomonas aeruginosa*	ATCC 15442; reference strain used in industrial testing, , originally isolated from an animal room water bottle; ENV
199	*Pseudomonas aeruginosa*	MWF
200	*Pseudomonas aeruginosa*	TC
202	*Pseudomonas aeruginosa*	LAC; 2012; EU
203	*Pseudomonas spp.*	PC; 2012
204	*Pseudomonas aeruginosa*	DWL; 2012
205	*Pseudomonas putida*	DWL; 2012; EU
206	*Pseudomonas pseudoalcaligenes*	LAC; 2012; EU
260	*Pseudomonas aeruginosa*	FWS; 2014; Brazil
261	*Pseudomonas aeruginosa*	FWS; 2012; Brazil
262	*Pseudomonas aeruginosa*	FWS; 2012; Brazil
263	*Pseudomonas aeruginosa*	FWS; 2014; Brazil
264	*Pseudomonas aeruginosa*	FWS; 2012; Brazil
265	*Pseudomonas aeruginosa*	FWS; 2012; Brazil
266	*Staphylococcus succinus*	FWS; 2012; Brazil

Footnotes: SC, surface cleaner; DWL, dish wash liquid; LAC, liquid abrasive cleaner; PC, personal care; RM, raw materials; Misc, miscellaneous; FWS, fabric wash; MWF, metal working fluid; TC, timber care; ENV, environmental isolation source

Table 2.4 Contaminated HPC-products sampled for bacterial growth

Product	Production year	Location	Isolate(s)
Dish Wash Liquid			
DWL1[a]	2000	-	RW182
DWL2[a]	1997	-	RW180
DWL3[a]	1998	-	RW181
DWL4[a]	2005	-	RW183
DWL5[a]	2013	-	*
DWL6	2009	-	RW172
DWL7	2011	Indonesia	RW173
DWL8	2010	Italy	RW174
DWL9	2009	Malaysia	RW175
DWL10	2010	Indonesia	RW176
DWL11	2010	Indonesia	RW177
DWL12a	2010	Malaysia	RW178
DWL12b	2010	Malaysia	RW179
DWL13	2006	-	RW193
DWL14	2011	-	RW195
DWL15	2006		RW184
Surface cleaner			
SC1[a]	2010	-	RW192
SC2[a]	-	-	RW185
SC3[a]	2013	-	*
SC4	-	-	RW186
SC5	2011	-	RW190
SC6	2011	-	RW191
SC7	2010		RW192
Personal Care			
PC1[a]	2000	-	RW189
PC2[a]	1996	-	RW188
PC3	2002	-	RW187
PC4	2003	-	RW194
Fabric Wash			
FWS1[a]	2012	Brazil	
FWS2[a]	2012	Brazil	*
FWS3[a]	2013	-	
Wood Cleaner			
WC1[a]	2013	-	*

[a]Products included in bacterial diversity investigations in Chapter 4; *Isolates from these products not in the collection, products used only in diversity investigations in Chapter 4.

2.8.3. Comparison of five selective media

A comparison of five selective agars for the detection of *P. aeruginosa* was performed. The five agars included in the study were PCN, PIA, ChromID Pa, Z-agar and mZ-agar (further details of the agars are given in section 2.2).

2.8.3.1. Strain panel for the comparative study

A strain panel comprising 148 strains including 58 *P. aeruginosa* and 90 non-*P. aeruginosa* strains was assembled for the agar comparison study (full details in Table 2.5). All *P. aeruginosa* isolates were confirmed as *P. aeruginosa* by the use of a species-specific PCR (*oprL* PCR; De Vos *et al.* 1997). Non-*P. aeruginosa* strains belonged to other *Pseudomonas* species, other Gram-negative species and Gram-positive species. Strains originated from clinical, environmental and industrial sources. Ninety strains were obtained from the collection held at Cardiff University (including those from the EuroCareCF panel) whilst 58 strains were supplied by industry or recovered from industrial sources.

2.8.3.2. Comparison of selective media

Overnight cultures of strains were diluted to an optical density (OD) of 1 at 600 nm (corresponding to approximately 10^8 cfu/ml) and 200 µl of the diluted culture pipetted into separate wells of a 96 well micro-titre plate. The cultures were replica plated, using a 48 pin replicator (approximately 10^5 cfu/ml transferred) from the 96 well plate onto TSA, PCN, PIA, chromID Pa, Z-agar and modified Z-agar in triplicate. All agar plates were incubated at 37°C and 42°C for 72 hours, except the TSA plates for the non-*P. aeruginosa* panel which were incubated at 30°C for 72 hours, and the Z-agar and modified Z-agar plates which were only incubated at 37°C. Results were recorded at 24 and 72 hours. Positive results on chromID Pa were originally intended to be taken as growth accompanied by a red/pink/purple colouration as described previously (Laine *et al.* 2009), but this interpretation was modified to also incorporate strains that displayed a pink-brown colour. Pigment production (pyocyanin and pyoverdin) by *P. aeruginosa* strains on TSA, PCN and PIA was recorded at 37°C. Growth on any of the other media was taken as a positive result, regardless of the presence/absence of pigment production.

2.8.3.3. Evaluation criteria

The sensitivity and specificity of the agars were calculated as follows. The sensitivity was taken as the number of *P. aeruginosa* strains identified as positive for growth on the media, divided by the total number of *P. aeruginosa* strains screened; the probability of a true positive. The specificity was taken as the number of non-*P. aeruginosa* strains negative for growth on the media, divided by the total number of non-*P. aeruginosa* strains screened; the probability of a true negative (Lalkhen and McCluskey 2008). Positive and negative predictive values (PPV/NPV) were calculated to determine the proportions of true positives and negatives for the study population. The PPV was taken as the number of *P. aeruginosa* strains identified as positive for growth on the media, divided by the total number of strains (both *P. aeruginosa* and non-*P. aeruginosa*) positive for growth. The NPV was taken as the number of non-*P. aeruginosa* strains negative for growth on the media, divided by the total number of strains (both *P. aeruginosa* and non-*P. aeruginosa*) which were negative for growth (Lalkhen and McCluskey 2008).

2.8.4. Acetamide media as enrichment media for *P. aeruginosa*

The Bioscreen C was used to examine the growth dynamics of *P. aeruginosa* and non-*P. aeruginosa* strains in Z-broth supplemented with different concentrations of acetamide, ranging from 1 to 50 g/L, as the selective agent (growth curve analysis was set up as described in section 2.5). Two experiments were performed to investigate growth over 72 hours at 37°C, as described in section 3.2.3 with varying acetamide concentrations and control isolates evaluated in each experiment. Where applicable, to examine differences between the maximum ODs reached at different acetamide concentrations for different strains, a Welch's 2-sample t-test for normally distributed data with unequal variances was performed in R statistical software.

Table 2.5 Strain collection used in the comparative agar study

Species (strain)	**Isolation source and other information**	**Supplied by**
Pseudomonas aeruginosa		
PAK-SR	CLIN; Streptomycin resistant parent of Fla-/Pil- mutants of PAK	Cardiff
PAO1	CLIN; First *P. aeruginosa* strain to be genome sequenced	Cardiff
C3790	CLIN, CF; Melbourne epidemic strain 1 (AES-1)	Cardiff
C3803	CLIN, CF; Melbourne epidemic strain 2 (AES-2)	Cardiff
P10119	CLIN, CF; Clone C epidemic strain	Cardiff
P9245	CLIN, CF; Midlands epidemic strain 1 (Mid1)	Cardiff
LES B58	CLIN, CF; Liverpool epidemic strain LES B58	Cardiff
NCTC 12903; ATCC 27853	CLIN; Antibiotic efficacy testing reference strain	Cardiff
C3719	CF; Manchester epidemic strain (MES)	Cardiff
N19	CLIN; Urinary tract infection (catheter) isolate	Cardiff
PA5	CLIN; Chronic prostatitis isolate (2006)	Cardiff
C259	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C132	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C1138	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C158	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C2957	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C4278	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C1698	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C511	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C2846	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C3128	CLIN, CF isolate; (Speert *et al.* 2002)	Cardiff
PA0025	CLIN	Cardiff
RW140	IND, isolated 1998	Industry
RW168	IND, isolated 1993	Industry
RW172	IND, isolated 2009	Industry
RW179	IND, isolated 2010	Industry
RW180	IND, isolated 1997	Industry
RW181	IND, isolated 1998	Industry
RW184	IND, isolated 2006	Industry
RW188	IND, isolated 1996	Industry
RW196	IND; ATCC 13388; reference strain used in industrial testing	Industry
RW197	IND; ATCC 10145; reference strain used in industrial testing	Industry
RW198	IND; ATCC 15442; reference strain used in industrial testing	Industry
RW199	IND; date of isolation unknown	Industry
RW200	IND; date of isolation unknown	Industry
RW113	IND, isolated 2011	Industry
RW130	IND, isolated 2010	Industry
RW133	IND, isolated 2011	Industry
RW135	IND, isolated 2012	Industry
RW137	IND, isolated 2011	Industry
RW138	IND, isolated 2001	Industry
RW147	IND, isolated 1996	Industry
RW149	IND, isolated 2004	Industry
RW151	IND; ATCC 9027; reference strain used in industrial testing	Industry
RW174	IND, isolated 2010	Industry
RW175	IND, isolated 2010	Industry
RW187	IND, isolated 2002	Industry
RW189	IND, isolated 2000	Industry
RW192	IND, isolated 2011	Industry
RW176	IND, isolated 2010	Industry
RW109	IND; date of isolation unknown	Industry
RW110	IND; date of isolation unknown	Industry
MB2.2	ENV strain; Domestic isolate from a washing machine in Cardiff, Autumn 2011	Domestic isolates
LM1.1	ENV strain; Domestic isolate from a kitchen sink in Cardiff, Autumn 2011	Domestic isolates

PA0048	Environmental isolate	Cardiff
PA0301	Environmental isolate	Cardiff
BCC0432	Environmental isolate	Cardiff
PA0003	Environmental isolate	Cardiff
Achromobacter xylosoxidans		
BCC1386	CLIN	Cardiff
LMG 1863	CLIN (EuroCareCF panel)	Cardiff
Acinetobacter species		
ESH#749	IND; date of isolation unknown	Industry
Bacillus species		
RW#162	IND; isolated 2009	Industry
Burkholderia ambifaria		
AMMD[a]	ENV; genome available	Cardiff
MC40-6	CLIN; genome available	Cardiff
MEX-5	ENV; genome available	Cardiff
Burkholderia cenocepacia		
AU1054[a]	CLIN; genome available	Cardiff
HI2424[a]	ENV; genome available	Cardiff
J2315; LMG 16656[a]	CLIN; genome available	Cardiff
LMG 12614[a]	CLIN (EuroCareCF panel)	Cardiff
LMG 16654[a]	CLIN (EuroCareCF panel)	Cardiff
LMG 16656[a]	CLIN (EuroCareCF panel)	Cardiff
LMG 18829[b]	CLIN (EuroCareCF panel)	Cardiff
LMG 18830	CLIN (EuroCareCF panel)	Cardiff
MC0-3[a]	ENV; genome available	Cardiff
RW#156[b]	IND; date of isolation unknown	Industry
Burkholderia cepacia		
LMG 1222[a]	CLIN (EuroCareCF panel)	Cardiff
***Burkholderia cepacia* complex**		
ESH#751[b]	IND; date of isolation unknown	Industry
ESH#750[a]	IND; date of isolation unknown	Industry
Burkholderia contaminans		
LMG 23255[a]	CLIN; genome available	Cardiff
Burkholderia dolosa		
LMG 18943	CLIN (EuroCareCF panel)	Cardiff
Burkholderia gladioli		
LMG 18157	CLIN (EuroCareCF panel)	Cardiff
Burkholderia lata		
383; ATCC 17660; LMG 22485	ENV; genome available	Cardiff
Burkholderia multivorans		
ATCC 17616	ENV; genome available	Cardiff
C1576	CLIN; genome available	Cardiff
LMG 13010	CLIN (EuroCareCF panel)	Cardiff
LMG 16660[a]	CLIN (EuroCareCF panel)	Cardiff
LMG 16775[a]	CLIN (EuroCareCF panel)	Cardiff
Burkholderia phymatum		
BCC1607[a]	ENV; genome available	Cardiff
Burkholderia phytofirmans		
BCC 1604	ENV; genome available	Cardiff
Burkholderia pyrrocinia		
LMG 21824[a]	CLIN (EuroCareCF panel)	Cardiff
Burkholderia stabilis		
LMG 14294[b]	CLIN (EuroCareCF panel)	Cardiff
Burkholderia vietnamiensis		
G4; ATCC 53617[a]	ENV; genome available	Cardiff
LMG 18835	CLIN (EuroCareCF panel)	Cardiff
RW#152	IND; isolated 2002	Industry
Escherichia coli		

NCTC 12241; ATCC 25922	CLIN; Antibiotic efficacy testing reference strain	Cardiff
***Halomonas* species**		
RW#171	IND; isolated 1997	Industry
Ochrobactrum anthropi		
RW#160	IND; isolated 1997	Industry
Pandoraea apista		
BCC0125	CLIN	Cardiff
LMG 16407	CLIN (EuroCareCF panel)	Cardiff
Pandoraea pnomenusa		
BCC0092; C7351	CLIN	Cardiff
BCC0580; C8029	CLIN	Cardiff
LMG 18087	CLIN (EuroCareCF panel)	Cardiff
Pandoraea pulmonicola		
BCC0150; FC0330	CLIN	Cardiff
LMG 18106	CLIN (EuroCareCF panel)	Cardiff
Pandoraea sputorum		
BCC0139	CLIN	Cardiff
BCC0212	CLIN	Cardiff
BCC0219	CLIN	Cardiff
LMG 18819	CLIN (EuroCareCF panel)	Cardiff
Pantoea agglomerans		
RW#166	IND; isolated 2002	Industry
Pseudomonas alcaligenes		
LMG 1224	ENV; isolated from swimming pool water	Cardiff
Pseudomonas aureofaciens		
LMG 1245[b]	ENV; isolated from Maas River clay suspended in kerosene for three weeks	Cardiff
Pseudomonas fluorescens		
LMG 1794[a]	ENV; isolated from water-works tanks	Cardiff
RW#85	IND; date of isolation unknown	Industry
RW#89	IND; date of isolation unknown	Industry
Pseudomonas mendocina		
LMG 1223	ENV; isolated from soil	Cardiff
Pseudomonas oleovorans		
LMG 2229[a]	IND; isolated from metal working fluid	Cardiff
Pseudomonas pseudoalcalienes		
RW#111	IND; date of isolation unknown	Industry
Pseudomonas putida		
LMG 2257	ENV; soil	Cardiff
RW#153[b]	IND; isolated 2003	Industry
RW#154	IND; isolated 2003	Industry
RW#157	IND; isolated 2010	Industry
RW#158	IND; isolated 2003	Industry
RW#159	IND; isolated 2006	Industry
RW#161	IND; isolated 2005	Industry
RW#205[b]	IND; isolated 2012	Industry
***Pseudomonas* species**		
RW#112[b]	IND; date of isolation unknown	Industry
RW#155	IND; isolated 2003	Industry
RW#163	IND; isolated 2004	Industry
RW#164	IND; isolated 2010	Industry
RW#165[b]	IND; isolated 2000	Industry
RW#167	IND; isolated 2002	Industry
RW#169	IND; isolated 2008	Industry
RW#206	IND; isolated 2012	Industry
Ralstonia insidiosa		
LMG 18101	CLIN (EuroCareCF panel)	Cardiff
Ralstonia mannitolilytica		
BCC1391	CLIN	Cardiff

LMG 18103	CLIN (EuroCareCF panel)	Cardiff
Ralstonia picketii		
LMG 18088	CLIN (EuroCareCF panel)	Cardiff
BCC0039	CLIN	Cardiff
BCC1392	CLIN	Cardiff
Serratia marcescens		
LMG 2792	ENV	Cardiff
LMG 25713	ENV	Cardiff
ATCC 14756	Isolation source unknown; test, control and bioassay strain	Cardiff
RW#168.2	IND; date of isolation unkown	Industry
Staphylococcus aureus		
ATCC 6538	CLIN	Cardiff
NCTC 12981[a]	CLIN	Cardiff
Staphylococcus epidermidis		
ATCC 12228	CLIN	Cardiff
Stenotrophomonas maltophilia		
BCC1394	CLIN	Cardiff
***Stenotrophomonas* species**		
ESH#758	IND; date of isolation unknown	Industry

[a]Strains recorded as positive on chromID Pa after incubation at 37°C for 24 hours, but negative on chromID Pa after incubation at 42°C for 24 hours

[b]Strains recorded a negative on chromID Pa after incubation at 37°C for 24 hours, but positive on chromID Pa after incubation at 42°C for 24 hours

LMG, Belgian co-ordinated collections of micro-organisms, Gent; ATCC, America type culture collection; NCTC, National collection of type cultures, Public Health England; BCC, Cardiff strain collection; ESH#/RW#, held in ESH/RW collections at Cardiff, originally supplied by industry; CLIN, clinical; CLIN CF, Cystic Fibrosis; ENV, environmental; IND, industrial

2.9. Culture-independent detection methods

2.9.1. Primer design for novel *P. aeruginosa*-specific PCRs

2.9.1.1. Primer design for PCR amplification of the *phzS* gene

The sequences of the *phzS* genes from 9 *P. aeruginosa* strains and *phzS* gene orthologs from 6 other bacterial species were obtained from the integrated microbial genomes (IMG) database (Markowitz *et al.* 2012). The 7 *phzS* orthologs from other bacterial species within the Class *Actinobacteria* were the top hits of an IMG database search using Basic Local Alignment Search Tool (BLAST) algorithms with the search input as the *P. aeruginosa* PA01 *phzS* gene complete sequence. The *phzS* gene and gene ortholog sequences were aligned with Clustal W2 (Larkin *et al.* 2007) and viewed using BioEdit Sequence Alignment Editor (Hall 1999). Two sets of primers (main and nested), specific for *P. aeruginosa* strains, were designed based on these alignments (Table 2.6).

2.9.1.2. Primer design for PCR amplification of the genes within the *ami* operon

The organisation of the *ami* operon of *P. aeruginosa* strains and orthologous operons in other bacterial species was viewed using the 'gene ortholog neighbourhood' viewer tool of the IMG database. The sequences of the *amiL-amiE* intergenic spacer region in 9 *P. aeruginosa* strains and orthologous sequences in other *Pseudomonas* species were obtained from the IMG database. In addition, the *amiE* gene sequences of 9 *P.aeruginosa* strains and *amiE* or orthologous genes from 5 strains of other *Pseudomonas* species were obtained from the IMG database and the *Pseudomonas* genome database (Winsor *et al.* 2009). Sequences were aligned using Clustal W2 and viewed using BioEdit Sequence Alignment Editor. One set of primers specific for *P. aeruginosa*, was designed based on these alignments (Table 2.6).

2.9.2. PCR reaction conditions for *P. aeruginosa* specific PCRs

PCR reactions had a total volume of 25 µl containing 1X Coralload buffer, 1X Q-solution, 200 µm deoxynucleoside triphosphates (dNTPs), 1.6 µm each primer, 1 U *Taq* polymerase and 2 µl DNA template (approximately 20 ng). If using DNA extracted from HPC-products, 6 µl was used as the template. All reagents were supplied by Qiagen. Primer pairs and PCR cycles for the different target genes are shown in Tables 2.6 and 2.7, respectively. For nested PCR reactions 2 µl of product from the first round of PCR was taken as the template for the second PCR reaction.

PCR cycles were performed on a c1000™ Thermal Cycler (Bio-Rad), and the annealing temperature optimised for specificity towards *P. aeruginosa*. Gel electrophoresis was performed on 1.5% (w/v) agarose gels stained with SafeView (10 µl SafeView per 100 ml of agarose gel). PCR products were visualised with a UV transilluminator and gel images captured using GeneSnap Software.

2.9.3. Evaluation of *phzS* and *ami* operon PCRs

The sensitivity and specificity of the *phzS* and *ami* operon PCR was evaluated using a panel comprising 60 *P. aeruginosa* and 65 non-*P. aeruginosa* strains (Table 2.8). To further evaluate the *phzS* PCR, four strains from a previous study (Finnan *et al.* 2004) that were found to be negative for the *phzS* gene were included in the *phzS* PCR test panel (Table 2.8). The sensitivity was taken as the number of *P. aeruginosa* strains identified as positive for the *phzS* or *ami* operon PCRs, divided by the total number of *P. aeruginosa* strains screened; the probability of a true positive. The specificity was taken as the number of non-*P. aeruginosa* strains negative for the *phzS* or *ami* operon PCRs, divided by the total number of non-*P. aeruginosa* strains screened; the probability of a true negative (Lalkhen and McCluskey 2008).

Table 2.6 Primers for the PCR amplification of *P. aeruginosa* genes

Target gene	Primer pair	Primer sequence 5'>3'	Annealing temp (°C)	Product Size (bp)	Reference
oprL	PAL1	ATG GAA ATG CTG AAA TTC GGC	61	504	(De Vos *et al.* 1997)
	PAL2	CTT CTT CAG CTC GAC GCG ACG			
	PAoprL.6	GCC TTC CAG CAC TAC GCG CTG			
phzS	phzS.F	GGA AGC CGG CAA CGC CTA T	65	584	This study
	phzS.R	GCA GGA TCA ACT GGT TGC GGG T			
***phzS*(nested)**	phzSn.F	CGC GGC GAA CTG CAG ATG ATC	65	435	This study
	phzSn.R	CTC GTT GTC GAG CTG GCC GA			
amiL-amiE	amiE.F	CAT CAG GTC RTG CGC ATC AGC	65	588	This study
	amiE.R	GCC CTT CGG CCC KTC GCT			

Table 2.7 PCR cycles for the amplification of *P. aeruginosa* genes

PCR step		Temperature (°C)	Time (minutes)	Nº cycles
1	Initial DNA denaturation	94	3	1
2	DNA denaturation	94	0.5	30
3	Primer annealing	[a]	1	
4	Primer extension	72	1	
5	Final extension	72	5	1
6	Indefinite hold	10	-	-

[a] Annealing temperatures vary for different primer sets (see Table 2.6)

Table 2.8 Strain collection used for the evaluation of the *phzS* and *ami* operon PCRs

Species (strain)	**Isolation source and other information**	**Supplied by**
Pseudomonas aeruginosa		
PAK-SR	CLIN; Streptomycin resistant parent of Fla-/Pil- mutants of PAK	Cardiff
PAO1	CLIN; First *P. aeruginosa* strain to be genome sequenced	Cardiff
C3790	CLIN, CF; Melbourne epidemic strain 1 (AES-1)	Cardiff
C3803	CLIN, CF; Melbourne epidemic strain 2 (AES-2)	Cardiff
P10119	CLIN, CF; Clone C epidemic strain	Cardiff
P9245	CLIN, CF; Midlands epidemic strain 1 (Mid1)	Cardiff
LES B58	CLIN, CF; Liverpool epidemic strain LES B58	Cardiff
NCTC 12903; ATCC 27853	CLIN; Antibiotic efficacy testing reference strain	Cardiff
C3719	CF; Manchester epidemic strain (MES)	Cardiff
N19	CLIN; Urinary tract infection (catheter) isolate	Cardiff
PA5	CLIN; Chronic prostatitis isolate (2006)	Cardiff
C259	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C132	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C1138	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C158	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C2957	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C4278	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C1698	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C511	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C2846	CLIN, CF; (Mahenthiralingam *et al.* 1996)	Cardiff
C3128	CLIN, CF isolate; (Speert *et al.* 2002)	Cardiff
PA0025	CLIN	Cardiff
RW140	IND, isolated 1998	Industry
RW168	IND, isolated 1993	Industry
RW172	IND, isolated 2009	Industry
RW179	IND, isolated 2010	Industry
RW180	IND, isolated 1997	Industry
RW181	IND, isolated 1998	Industry
RW184	IND, isolated 2006	Industry
RW188	IND, isolated 1996	Industry
RW196	IND; ATCC 13388; reference strain used in industrial testing	Industry
RW197	IND; ATCC 10145; reference strain used in industrial testing	Industry
RW198	IND; ATCC 15442; reference strain used in industrial testing	Industry
RW199	IND; date of isolation unknown	Industry
RW200	IND; date of isolation unknown	Industry
RW113	IND, isolated 2011	Industry
RW129	IND; isolated 2010	Industry
RW130	IND, isolated 2010	Industry
RW133	IND, isolated 2011	Industry
RW135	IND, isolated 2012	Industry
RW137	IND, isolated 2011	Industry
RW138	IND, isolated 2001	Industry
RW147	IND, isolated 1996	Industry
RW149	IND, isolated 2004	Industry
RW151	IND; ATCC 9027; reference strain used in industrial testing	Industry
RW174	IND, isolated 2010	Industry
RW175	IND, isolated 2010	Industry
RW187	IND, isolated 2002	Industry
RW189	IND, isolated 2000	Industry
RW192	IND, isolated 2011	Industry
RW194	IND; isolated 2011	Industry
RW176	IND, isolated 2010	Industry

RW109	IND; date of isolation unknown	Industry
RW110	IND; date of isolation unknown	Industry
MB2.2	ENV strain; Domestic isolate from a washing machine in Cardiff, Autumn 2011	Domestic isolates
LM1.1	ENV strain; Domestic isolate from a kitchen sink in Cardiff, Autumn 2011	Domestic isolates
PA0048	Environmental isolate	Cardiff
PA0301	Environmental isolate	Cardiff
BCC0432	Environmental isolate	Cardiff
PA0003	Environmental isolate	Cardiff
CF194*	(Finnan *et al.* 2004)	University College Cork (UCC)
CF198*	(Finnan *et al.* 2004)	UCC
CF242*	(Finnan *et al.* 2004)	UCC
BR227*	(Finnan *et al.* 2004)	UCC
Achromobacter xylosoxidans		
LMG 1863	CLIN (EuroCareCF panel)	Cardiff
Acinetobacter species		
ESH#749	IND; date of isolation unknown	Industry
Bacillus species		
RW#162	IND; isolated 2009	Industry
Burkholderia ambifaria		
AMMD[a]	ENV; genome available	Cardiff
Burkholderia cenocepacia		
J2315; LMG 16656[a]	CLIN; genome available	Cardiff
RW#156[b]	IND; date of isolation unknown	Industry
***Burkholderia cepacia* complex**		
ESH#751[b]	IND; date of isolation unknown	Industry
ESH#750[a]	IND; date of isolation unknown	Industry
Burkholderia contaminans		
LMG 23255[a]	CLIN; genome available	Cardiff
Burkholderia dolosa		
LMG 18943	CLIN (EuroCareCF panel)	Cardiff
Burkholderia gladioli		
LMG 18157	CLIN (EuroCareCF panel)	Cardiff
Burkholderia lata		
383; ATCC 17660; LMG 22485	ENV; genome available	Cardiff
Burkholderia multivorans		
ATCC 17616	ENV; genome available	Cardiff
Burkholderia phymatum		
BCC1607[a]	ENV; genome available	Cardiff
Burkholderia phytofirmans		
BCC 1604	ENV; genome available	Cardiff
Burkholderia pyrrocinia		
LMG 21824[a]	CLIN (EuroCareCF panel)	Cardiff
Burkholderia stabilis		
LMG 14294[b]	CLIN (EuroCareCF panel)	Cardiff
Burkholderia vietnamiensis		
G4; ATCC 53617[a]	ENV; genome available	Cardiff
RW#152	IND; isolated 2002	Industry
***Enterobacter* species**		
ESH#752	IND; date of isolation unknown	Industry
Enterobacter gergoviae		
ESH#753	IND; date of isolation unknown	Industry
Escherichia coli		
NCTC 12241; ATCC 25922	CLIN; Antibiotic efficacy testing reference strain	Cardiff
***Halomonas* species**		
RW#171	IND; isolated 1997	Industry
***Klebsiella* species**		

ESH#759	IND; date of isolation unknown	Industry
Ochrobactrum anthropi		
RW#160	IND; isolated 1997	Industry
Pandoraea apista		
LMG 16407	CLIN (EuroCareCF panel)	Cardiff
Pandoraea pnomenusa		
LMG 18087	CLIN (EuroCareCF panel)	Cardiff
Pandoraea pulmonicola		
LMG 18106	CLIN (EuroCareCF panel)	Cardiff
Pandoraea sputorum		
LMG 18819	CLIN (EuroCareCF panel)	Cardiff
Pantoea agglomerans		
RW#166	IND; isolated 2002	Industry
Pseudomonas alcaligenes		
LMG 1224	ENV; isolated from swimming pool water	Cardiff
Pseudomonas aureofaciens		
LMG 1245[b]	ENV; isolated from Maas River clay suspended in kerosene for three weeks	Cardiff
Pseudomonas fluorescens		
LMG 1794[a]	ENV; isolated from water-works tanks	Cardiff
Pseudomonas mendocina		
LMG 1223	ENV; isolated from soil	Cardiff
Pseudomonas oleovorans		
LMG 2229[a]	IND; isolated from metal working fluid	Cardiff
Pseudomonas pseudoalcalienes		
RW#111	IND; date of isolation unknown	Industry
Pseudomonas putida		
LMG 2257	ENV; soil	Cardiff
RW#153[b]	IND; isolated 2003	Industry
RW#154	IND; isolated 2003	Industry
RW#157	IND; isolated 2010	Industry
RW#158	IND; isolated 2003	Industry
RW#159	IND; isolated 2006	Industry
RW#161	IND; isolated 2005	Industry
RW#205[b]	IND; isolated 2012	Industry
***Pseudomonas* species**		
RW#112 [b]	IND; date of isolation unknown	Industry
Ralstonia insidiosa		
LMG 18101	CLIN (EuroCareCF panel)	Cardiff
Ralstonia mannitolilytica		
LMG 18103	CLIN (EuroCareCF panel)	Cardiff
Ralstonia picketii		
LMG 18088	CLIN (EuroCareCF panel)	Cardiff
Staphylococcus aureus		
ATCC 6538	CLIN	Cardiff
NCTC 12981[a]	CLIN	Cardiff
Staphylococcus epidermidis		
ATCC 12228	CLIN	Cardiff
Stenotrophomonas maltophilia		
BCC1394	CLIN	Cardiff
***Stenotrophomonas* species**		
ESH#758	IND; date of isolation unknown	Industry

*Strains only used for the evaluation of the *phzS* PCR

LMG, Belgian co-ordinated collections of micro-organisms, Gent; ATCC, America type culture collection; NCTC, National collection of type cultures, Public Health England; BCC, Cardiff strain collection; ESH#/RW#, held in ESH/RW collections at Cardiff, originally supplied by industry; CLIN, clinical; CLIN CF, Cystic Fibrosis; ENV, environmental; IND, industrial

2.10. Genotyping techniques and investigation of strain diversity

2.10.1. PCR-fingerprinting: Random Amplified Polymorphic DNA (RAPD) typing

RAPD typing was adapted from Mahenthiralingam *et al.* (Mahenthiralingam *et al.* 1996) and Fothergill *et al.* (Fothergill *et al.* 2010). PCR reaction mixtures had a total volume of 25 µl, comprising 1 x PCR buffer, 1 x Q-solution, 3 mM $MgCl_2$, 200 µm dNTPs, 1.6 µM primer 272 (5′-AGCGGGCCAA-3′), 1 U *Taq* DNA polymerase and 2 µl template genomic DNA (approximately 20 ng). All PCR reagents were supplied by Qiagen and PCR reactions were carried out on a c1000™ Thermal Cycler with the cycles shown in Table 2.9. 1µl of RAPD-PCR product was loaded onto a DNA 7500 chip and analysed by microfluidics using an Agilent 2100 Bioanalyzer, according to the manufacturer's instructions. Cluster analysis of the RAPD-PCR profiles was performed using the Gel Compar package within Bionumerics 6.6 software (Applied Maths). A Pearson correlation similarity coefficient with a UPGMA dendrogram type was used to determine similarity between RAPD profiles. Isolate profiles displaying ≥80% profile similarity were considered as a single RAPD type (White *et al.* 2011). The selection of *P. aeruginosa* isolates associated with industry and subjected to RAPD typing analysis is given in Table 2.10.

Table 2.9 RAPD-PCR cycles

PCR step		Temperature (°C)	Time (minutes)	Nº cycles
1	DNA denaturation	94	5	4
2	Primer annealing	36	5	
3	Primer extension	72	5	
4	DNA denaturation	94	1	30
5	Primer annealing	36	1	
6	Primer extension	72	2	
7	Final extension	72	6	
8	Indefinite hold	10	-	-

Table 2.10 *P. aeruginosa* strains associated with industry used in RAPD-PCR typing

RW#	Comment
109	Challenge test strain used in preservative efficacy testing
110	Challenge test strain used in preservative efficacy testing
113	SC; 2011
114	SC; 2011
115	SC; 2011
116	SC; 2011
117	DWL; 2010; Indonesia
118	DWL; 2010; Indonesia
119	DWL; 2010; Italy
120	DWL; 2010; Italy
121	DWL; 2010; Italy
122	DWL; 2009; Italy;
123	DWL; 2011; Malaysia
124	DWL; 2009; Italy
125	DWL; 2009; Italy
126	DWL; 2009; Italy
127	DWL; 2010; Indonesia
128	DWL; 2010; Italy
129	DWL; 2010; Italy
130	DWL; 2010; Italy
131	DWL; 2010; Italy
132	DWL; 2010; Italy
133	DWL; 2010; Thailand
134	DWL; 2010; Thailand
135	DWL; 2010; Indonesia
136	DWL; 2010; Indonesia
137	DWL; 2010; Italy
138	LAC; 2001
139	LAC; 2001
140	DWL
143	DWL
144	DWL
145	DWL; 2010
146	DWL; 2004; Italy
147	PC
148	PC
149	PC
150	PC
151	strain ATCC 9027 used in period after opening studies
168.2	FWS
172	DWL; 2009
173	DWL; 2011
174	DWL; 2010
175	DWL; 2010
176	DWL; 2010
177	DWL; 2010
178	DWL; 2010
179	DWL; 2010
180	DWL
181	DWL
182	DWL
184	DWL
185	SC
187	PC
188	PC

189	PC
190	SC
191	SC
192	SC
193	PC; 2003
194	DWL; 2011; Trinidad and Tobago
195	DWL; 2006
196	ATCC 13388; reference strain used in industrial testing (ISO 846C); origin unknown
197	ATCC 10145; reference strain used in industrial testing,
198	ATCC 15442; reference strain used in industrial testing, , originally isolated from an animal room water bottle; ENV
199	MWF
200	TC
202	LAC; 2012; EU
204	DWL; 2012

SC, surface cleaner; DWL, dish wash liquid; LAC, liquid abrasive cleaner; PC, personal care; RM, raw materials; Misc, miscellaneous; FWS, fabric wash; MWF, metal working fluid; TC, timber care; ENV, environmental isolation source

2.10.2. Clondiag Array-Tube typing

The Clondiag Array Tube (AT) *P. aeruginosa* genotyping kit (Alere Technologies Gmbh, Germany) was used to genotype selected *P. aeruginosa* strains according to the manufacturer's instructions. This genotyping method samples the core genome (via the detection of 13 single nucleotide polymorphisms) and the accessory genome (via the detection of 39 variable genetic markers). Interpretation of the presence or absence of the SNPs representing the core genome results in a 16-digit binary genotype, that is expressed as a four letter hexadecimal code. The hexadecimal code types can be compared to a database of previously documented strain types and used in population structure analyses, for example with the eBURST (enhanced version of based upon related sequence types) algorithm (Feil *et al.* 2004). The relatedness of the *P. aeruginosa* strains was determined by comparison to a 917-strong database of clinical and environmental strains [a subset of the database of (Cramer N. *et al.* 2012)] using the eBURST algorithm (http://eburst.mlst.net) (Feil *et al.* 2004).

2.10.3. Whole genome sequence-based methods

2.10.3.1. Genome sequencing: DNA preparation, sequencing technology and draft genome assembly

Genomic DNA extraction from pure bacterial cultures and total was achieved using the Maxwell® 16 instrument as described in section 2.6.3. Quantitation and quality control of the extracted DNA was performed as described in section 2.6.4. Genomic library preparation and DNA sequencing was performed at the Oxford Genomics Centre of the Welcome Trust Centre for Human Genetics. DNA sequencing was achieved using 100-bp paired-end Illumina HiSeq2000 technology. *De novo* assembly of genomes from the paired-end sequence data was performed using SPAdes Genome Assembler v3.5.0 (Bankevich *et al.* 2012), genome assembly statistics are given in Table 2.11. Sequences were uploaded to the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena).

Table 2.11 SPAdes genome assembly statistics for the 24 *P. aeruginosa* genome sequenced strains

Strain (RW#)	Other strain designations	Genome assembly information				
		Number of contigs[a]	N50[a]	Fold sequence coverage	Assembly length (Mb) [a]	GC%[a]
11	NCTC 12903; ATCC 27853	89	296553	25.79	6.76	66.15
18		87	189759	26.57	6.60	66.18
27	A128	80	302083	20.47	6.47	66.3
30	A128a	71	340995	24.38	6.32	66.4
99	MB2.2	84	271043	24.72	6.71	66.26
109		201	134144	19.60	7.54	65.2
110		52	373781	29.90	6.58	66.33
130		187	125540	28.10	7.63	65.31
131		182	125540	31.19	7.58	65.33
138		140	165596	28.74	7.10	65.78
146		204	108166	21.94	7.65	65.29
149		161	157029	22.91	7.21	65.73
151	ATCC 9027	139	171689	24.20	6.82	65.97
168		65	273926	26.33	6.48	66.35
172		152	183227	23.14	7.45	65.34
176		167	142369	30.55	7.35	65.4
184		259	133442	20.95	7.06	65.58
192		113	211670	30.15	6.70	65.93
196	ATCC 13388; NCTC 8060	125	206633	34.99	6.62	66.16
198	ATCC 15442	86	264882	35.09	6.68	66.25
199		218	137755	34.94	7.02	65.78
200		89	210395	37.30	6.45	66.38
202		59	319926	37.77	6.58	66.33
204		79	272723	31.52	6.78	65.91

[a]All statistics are based on contigs of size >= 500 bp

2.10.3.2. BIGSdb for analysis of genomic data

Assembled genomic data were deposited in the PubMLST database, supported by the Bacterial Isolate Genome Sequence Database (BIGSdb) software (Jolley and Maiden 2010). The BIGSdb software has the capacity not only to store sequence data (ranging from single sequence reads to whole genomes) but can link entries to information about isolate provenance and phenotype. In addition BIGSdb is a powerful tool for investigations into population genomics. The Genome Comparator module within BIGSdb facilitates gene-by-gene analyses through the use of existing genotyping schemes defined within the database, such as Multilocus Sequence Typing (MLST) or ribosomal MLST (rMLST), as well as schemes defined by the user. For example, whole genome MLST (wgMLST) or a scheme involving genes involved in certain biochemical pathways, can be implemented using an annotated reference genome as the comparator for analysis (Jolley and Maiden 2010). BIGSdb can build up profiles of alleles at each locus for genotyping schemes, allowing the identification of different alleles type for the isolates under investigation and further analysis through MLST profile comparisons (Jolley *et al.* 2012b).

2.10.3.3. Genome comparison

Using BIGSdb investigations into the strain diversity of *P. aeruginosa* were conducted with: (i) the 7 *P. aeruginosa* MLST loci (Curran *et al.* 2004); (ii) the 53 rMLST loci (Jolley *et al.* 2012a); and (iii) wgMLST with all of the loci defined in the *P. aeruginosa* UCBPP-PA14 genome annotation. Loci were compared between *P. aeruginosa* strains using the Genome Comparator tool which outputs MLST profiles and a distance matrix based on the number of variable alleles. Phylogenetic networks were generated from the distance matrices using the NeighbourNet algorithm (Bryant and Moulton 2004) in SplitsTree v4.13.1 (Huson and Bryant 2006). This algorithm can produce a net-like diagram supporting multiple plausible evolutionary pathways between sequences, which may arise due through recombinational events such as horizontal gene transfer (Cody *et al.* 2013)

2.11. Microbial diversity analysis

2.11.1. Ribosomal Intergenic Spacer Analysis (RISA) PCR and profile processing

Bacterial diversity profiling was carried out using RISA, adapted from (Baxter *et al.* 2013). RISA PCRs reaction mixtures had a final volume of 25 µl comprising 1 x PCR buffer, 1 x Q-solution, 200 µm deoxynucleoside triphosphates (dNTPs), 0.4 µm each RISA PCR primer (1406F: 5'-TGYACACACCGCCCGT-3' and 23SR: 5'-GGGTTBCCCCATTCRG-3'; (Fisher and Triplett 1999), 1 U *Taq* polymerase. The amount of template DNA used was 2 µl for DNA extracted from pure cultures and 6 µl for DNA extracted from HPC-products. All PCR reagents were supplied by Qiagen and PCR reactions were carried out on a c1000™ Thermal Cycler with the cycles shown in Table 2.12. RISA-PCR products (1µl) were loaded onto a DNA 7500 chip and analysed using an Agilent 2100 Bioanalyzer, according to the manufacturer's instructions. The number of bands observed in each RISA-PCR profile was taken as an indicator of sample diversity. To aid the putative identification of contaminants, DNA from a pure bacterial culture of *P. aeruginosa* PAO1 was subjected to RISA and the online *In Silico* PCR tool was used to predict the size of ITS regions from likely contaminating species.

Table 2.12 RISA-PCR cycles

PCR step		Temperature (°C)	Time (minutes)	Nº cycles
1	Initial DNA denaturation	95	5	1
2	DNA denaturation	95	1	35
3	Primer annealing	54	0.5	
4	Primer extension	72	1	
5	Final extension	72	5	1
6	Indefinite hold	10	-	-

2.11.2. Bacterial 16S rRNA gene pyrosequencing and data processing

DNA extracted from home and personal care products (section 2.6.3) was used directly (without dilution) for 16S rRNA gene amplification and pyrosequencing as described by (Dowd *et al.* 2008), performed commercially at Research and Testing Laboratories Inc. (Lubbock, Texas, USA). The universal bacterial 16S rRNA primers 530F (5'-GTGCCAGCMGCNGCGG-3') and 1100R (5'-GGG TTN CGN TCG TTG-3') were used for amplification and the V4-V6 regions of the 16S rRNA gene sequenced using the Roche GS-FLX platform (Roche, Nutley, New Jersey).

A two-stage approach was used to analyse the resulting sequence data, similar to that described by (Bokulich *et al.* 2013). Firstly, the 16S rRNA sequences were analysed using the Mothur software package (Schloss *et al.* 2009) following the 454 SOP pipeline. Each sample data set was subsampled in Mothur to the lowest number of sequence reads found across the samples. Sequences were grouped into operational taxonomic units (OTUs) based on a 0.03 distance limit, which equated to 97% sequence similarity. Mothur outputs for OTU richness measured by observed species numbers (Sobs), the Shannon and Inverse Simpson diversity indices and Good's coverage estimates for the data were noted. Secondly, the RDP MultiClassifier script (Wang *et al.* 2007) was used to determine OTU taxonomies to the genus level, with manual searching of the RDP-II sequence database to corroborate assignments. Following taxonomic assignment, an OTU minimum abundance threshold of 10 was applied which removed OTUs represented fewer than 10 times across the dataset. Relative abundances of genera were calculated per sample.

2.12. Antimicrobial susceptibility profiling

2.12.1. Minimum inhibitory concentration (MIC) determination for individual preservatives

2.12.1.1. Broth MIC determination

The susceptibility of a panel of 40 *P. aeruginosa* strains isolated from CF individuals (10), and clinical (9), environmental (5), industrial (15) and unknown (1) sources was determined for 5 preservatives. Aqueous stock solutions of the 5 preservatives were diluted (serial doubling dilutions) in TSB to produce a range of concentrations: 0 – 0.01% CITMIT; 0 – 0.01% MIT; 0 – 0.08% BIT; 0 – 2.5% PHE; 0 – 0.1% CHX; and 0 – 0.4% BA. Dilutions of BA were prepared in TSB at pH 5.

Overnight cultures of *P. aeruginosa* were adjusted to an optical density (OD) of 1 at 600 nm (corresponding to approximately 10^8 cfu/ml) and 200 µl pipetted into separate wells of a 96-

well microplate, producing a masterplate for the panel. Using a 48-pin replicator, approximately 10^5 cfu from each well of the masterplate were inoculated into fresh 96-well microplates containing the preservative concentrations (200 µl of preservative concentration per well). Microplates were incubated shaking (150 rpm) for 24 hours at 37°C. After incubation the optical density of each well at 600 nm was recorded using the absorbance function of a microplate reader (Tecan Infinite® 200 PRO; Tecan UK Ltd., Reading, UK). The MIC was taken as the concentration of preservative at which there was an 80% reduction in OD from growth control wells containing TSB. Experiments were performed twice with different starting cultures and preservatives stock solutions to obtain biological replicates, each experiment having two technical replicates.

2.12.1.2. Statistical analysis of MIC values

The MIC data were analysed as discreet data using non-parametric statistical methods. The median of the four replicates was recorded to obtain a final MIC value for each strain for the different preservatives. Boxplots were generated using BoxPlotR to examine the distribution of the MIC data for each preservative. Comparisons between strain groups from different isolation sources were performed using a Kruskal-Wallis test and post-hoc Wilcoxon tests with Benjamini-Hochberg correction. The medians of the different groups were deemed statistically significantly different at the p=0.05 level.

2.12.2. Preservative combination testing

2.12.2.1. Checkerboard method for combination testing

A checkerboard method (Berenbaum 1978) was used to assess the susceptibility of a panel of 40 *P. aeruginosa* strains (detailed in Chapter 6, Table 6.1) to a combination of two preservatives or one preservative and one preservative enhancing agent. Each strain was exposed to dilutions of two agents diluted in TSB, in a 96 well plate format; one agent increased in concentration along the horizontal axis, the other increased along the vertical axis. The combinations that were tested were as follows: MIT and BIT; CITMIT and PAβN; PHE and CX3; PHE and CX7; PHE and VSC. The concentrations of preservatives and preservative enhancing agents that were tested are given in Table 2.13. The CITMIT blend was considered as one preservative for the purpose of these assays.

Microtitre plates containing the preservative dilutions were inoculated with approximately 10^5 cfu/ml of an overnight culture of *P. aeruginosa* PA14 per well. Plates were incubated at 37°C for 24 hours shaking at 150 rpm. The MIC was taken as the concentration of

preservative/preservative combination at which there was an 80% reduction in OD from growth control wells containing TSB.

Table 2.13 Preservative concentrations used in combination testing

Preservative/ Preservative enhancing agent concentration (%)							
MIT	BIT	CITMIT	PAβN	PHE	CX3	CX7	VSC
0	0	0	0	0	0	0	0
0.0001	0.0025	0.0005	10	0.02	0.1	0.1	1
0.00025	0.005	0.00075	25	0.04	0.25	0.25	2
0.0005	0.01	0.001	50	0.08	0.5	0.5	3
0.001	0.015	0.002	100	0.1	1	1	4
0.0025	0.02	0.003	250	0.2	2.5	2.5	5
0.005	0.025	0.004	500	0.4			
	0.03	0.005		0.8			
	0.04	0.01		1			

Footnotes: MIT, methylisothiazolinone; BIT, benzisothiazolinone; CITMIT, chloromethylisothiazolinone and methylisothiazolinone blend in the ratio 3:1; PAβN, L-Phe-Arg-β-napthylamide; PHE, phenoxyethanol, CX3, CX3 preservative enhancer; CX7, CX7 preservative enhancer; VSC, Velsan SC preservative enhancer.

2.12.2.2. Interpretation of checkerboard testing

The checkerboard test was interpreted using the fractional inhibitory concentration (FIC) index. The combined activity of two agents was calculated using the following formula:

$$FIC = FICA + FIC\ B = [A]/MICA + [B]/MICB$$

In this equation [A] and [B] are the concentrations of the two agents being tested and 'MICA' and 'MICB' are the MICs of the individual agents. The relationships between the two agents were described as follows: FIC <0.5, synergistic; FIC 0.5-1, additive; FIC 1-4, indifferent; FIC >4, antagonistic (Stanojevic *et al.* 2009). The lowest FIC index across the range of dilutions for both agents was reported.

2.13. Motility assays

All agar plates for motility assays were poured on an even surface and dried under laminar flow for 30 minutes. Agar concentrations were prepared by the addition of molecular biology grade agarose (Severn Biotech Ltd., UK) to either LB Broth (LB; Sigma-Aldrich Co. Ltd) or Basal Salts Medium (Hareland *et al.* 1975) supplemented with 0.4% (w/v) glucose (BSM-G). At least two biological replicates were performed per strain for each of the assays. Methods were modified from (Rashid and Kornberg 2000).

2.13.1. Swimming

Swimming motility was assessed by inoculating the surface of a 0.3% (w/v) LB agar plate with overnight culture using a sterile toothpick. Swimming plates were incubated overnight at 37°C for 16-18 hours, wrapped in a petri-dish bag to prevent dehydration. The diameter of the swimming zone was calculated by taking an average of two perpendicular measurements. Strains were scored as non-motile (diameter ≤ 5 mm), motile (diameter > 5 mm and ≤ 60 mm), or highly motile (diameter > 60 mm).

2.13.2. Swarming

Media used to assess swarming motility were 0.5% (w/v) LB agar and 0.5% (w/v) BSM-G agar. Swarming plates were surface inoculated with growth from an overnight culture using a sterile toothpick, and incubated overnight at 30°C for 16-18 hours, wrapped in a petri dish bag to prevent dehydration. The diameter of the swarming zone was calculated by taking an average of two perpendicular measurements. Strains were scored as non-motile (diameter ≤ 5 mm), motile (diameter > 5 mm and ≤ 60 mm), or highly motile (diameter > 60 mm).

2.13.3. Twitching

Twitching motility was assessed using 1% (w/v) LB agar plates. Using a sterile toothpick, twitch plates were stab inoculated to the base of the petri dish with overnight culture. Following overnight incubation for 16-18 hours at 37°C, zones of motility at the agar/petri dish interface were stained to improve visualisation, using a method modified from (McMichael 1992). Twitch plates were dried under laminar flow for 30 minutes and the agar removed prior to staining the dish with 0.5% (w/v) Coomassie brilliant blue R250 (Sigma-Aldrich Co. Ltd.; dissolved in an aqueous solution of 40% methanol and 10% (v/v) acetic acid) for 2 mins. Excess stain was washed off with an aqueous solution of 40% (v/v) methanol and 10% (v/v) acetic acid. The diameter of the twitching zone was calculated by taking an average of two perpendicular

measurements. Strains were scored as non-motile (diameter ≤ 5 mm), motile (diameter > 5 mm and ≤ 30 mm), or highly motile (diameter > 30 mm).

2.14. Adaptation to a preservative combination

2.14.1. Susceptibility a *P. aeruginosa* PA14 to a preservative combination

The *P. aeruginosa* strain PA14 was used in all of the adaptation assays. The preservative combination used was 0.0009% CITMIT, 0.22% DMDMH and 0.1% EDTA (referred to as CITMIT-DMDMH-EDTA). The preservatives (CITMIT and DMDMH) and preservative enhancing agent (EDTA) were made to these concentrations in TSB (taken as 100% CITMIT-DMDMH-EDTA) and further dilutions made in TSB. To determine the base-level susceptibility of *P. aeruginosa* PA14 to CITMIT-DMDMH-EDTA, growth in the presence of different dilutions of the combination was examined using the Bioscreen C instrument (set up described in section 2.5.1) over 48 hours. Alongside a TSB only growth control, the following concentrations of the preservative combination were tested: 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 25%, 50% and 100%. From this experiment, sub-inhibitory concentrations of CITMIT-DMDMH-EDTA were selected to start the planktonic and biofilm adaptation experiments.

2.14.2. Experimental evolution with planktonic growth

A method modified from Wong *et al.* (Wong *et al.* 2012) was followed to investigate the adaptation of planktonic growth of *P. aeruginosa* PA14 to CITMIT-DMDMH-EDTA. Approximately 10^5 cfu from an overnight culture of PA14 were inoculated into 10 tubes containing 3 ml of TSB, 10 tubes containing 3 ml of 2.5% strength CITMIT-DMDMH-EDTA in TSB, and a further 10 tubes with 3ml of 2.5% strength CITMIT-DMDMH-EDTA in TSB. One of the sets of replicates with the 2.5% strength CITMIT-DMDMH-EDTA was used as a growth control, whilst the other was a 'test' set where the concentration of CITMIT-DMDMH-EDTA was increased at each transfer during the experiment in 2.5% increments. Every 24 hours for 8 days, each of the 30 populations was propagated by transferring 3 µl of culture into 3 ml of fresh growth medium and incubated at 37°C. The populations grown in TSB only and in the 2.5% strength CITMIT-DMDMH-EDTA growth control were transferred in fresh TSB and 2.5% strength CITMIT-DMDMH-EDTA, respectively. The 'test' populations initially grown in 2.5% CITMIT-DMDMH-EDTA, were transferred into both 2.5% and 5% CITMIT-DMDMH-EDTA. After 24 hours, if there was growth in the 5% CITMIT-DMDMH-EDTA, the culture would be transferred into 5% and 7.5% CITMIT-DMDMH-EDTA, and so on. If there was not growth at 5% CITMIT-DMDMH-EDTA, a transfer would be made from growth from the 2.5% CITMIT-DMDMH-

EDTA into fresh 2.5% and 5% CITMIT-DMDMH-EDTA again. This transfer strategy was continued throughout the experiment to ensure growth was obtained for all 10 'test' populations. Freezer stocks were made of the 30 populations after 4 transfers and 8 transfers by adding 8% DMSO to the cultures and storing them at - 80°C.

2.14.3. Experimental evolution in a biofilm

To investigate adaptation of *P. aeruginosa* PA14 biofilms to CITMIT-DMDMH-EDTA, a method modified from Poltak and Cooper (Poltak and Cooper 2011) was used. Briefly, biofilms were grown on 7 mm diameter plastic beads (American Educational Products; sterilised by autoclaving at 121°C for 15 minutes) suspended in 5 ml growth medium exposed to gentle rocking (Bibby Stuart Platform Rocker STR6; 10 rev/min) at 37°C, and transferred into 5 ml of fresh growth medium every 48 hours for one month (14 transfers). Three conditions were studied: (1) A planktonic growth control with TSB as the growth medium; (2) A biofilm growth control with TSB as the growth medium and a bead for biofilm growth; and, (3) A biofilm test condition with TSB plus increasing concentrations of CITMIT-DMDMH-EDTA and a bead for biofilm growth. Two biological replicates were set up for each condition, with six technical replicates per biological replicate to allow for planktonic growth and a bead to be sampled on every third transfer day (6 samples).

To begin the experiment, tubes containing 5 ml TSB, 5 ml TSB plus one sterile plastic bead and 5 ml TSB with 7.5% strength CITMIT-DMDMH-EDTA plus one sterile plastic bead were inoculated with 10^5 cfu of an overnight culture of *P. aeruginosa* PA14. All cultures were grown rocking at 37°C. Every 48 hours a transfer was made: (1) Planktonic growth was diluted 1:100 into fresh TSB; (2) The bead from the biofilm growth control was transferred, using sterile tweezers, into 5 ml fresh TSB and a new bead to be colonised with biofilm growth; and (3) The bead from the biofilm test condition was transferred into either the same strength CITMIT-DMDMH-EDTA or a higher CITMIT-DMDMH-EDTA concentration (every third transfer the CITMIT-DMDMH-EDTA concentration increased as follows: 7.5%, 10%, 12.5%, 15%, 20%) containing a new bead. All three conditions were sampled on the start day, on every third transfer day, and the last day of the experiment; freezer stocks were made of planktonic growth from all three conditions by adding 8% DMSO and storing frozen at -80°C. In addition, total viable counts were performed on planktonic growth from condition (1) and for planktonic growth and colonised beads from conditions (2) and (3). To obtain total viable counts for colonised beads, beads were removed into 2 ml PBS, vortex mixed for 1 minute to remove biofilm growth, then the remaining suspension used. Freezer stocks were also made from the remaining suspension by adding 8%

DMSO and storing frozen at -80°C. Different colony morphologies from the total viable counts were recorded.

2.14.4. Preservative susceptibility profiling of ancestral and derivative isolates

The preservative susceptibilities of a selection of end-point isolates from the planktonic and biofilm experiments (Table 2.14) were determined in relation to the ancestral *P. aeruginosa* PA14 strain. The MICs of CITMIT, DMDMH and the preservative combination CITMIT-DMDMH-EDTA were calculated using broth dilution assays in 96 well microplates. Stock solutions of the preservatives and 100% CITMIT-DMDMH-EDTA were diluted in TSB to produce the range of concentrations listed in Table 2.15. Microplates containing the preservative dilutions were inoculated with approximately 10^5 cfu/ml of an overnight cultures of *P. aeruginosa* PA14 or derivate isolates per well. Plates were incubated at 37°C for 24 hours shaking at 150 rpm. The MIC was taken as the concentration of preservative/preservative combination at which there was an 80% reduction in OD from growth control wells containing TSB.

Table 2.14 Isolates from the adaptation experiments chosen for preservative susceptibility testing

Experimental conditions	Isolate(s)	Description/Comment
Pre-experiment		
	P. aeruginosa PA14	Ancestral strain for both experiments
Adaptation of planktonic growth to CITMIT-DMDMH-EDTA		
TSB growth control	TSB.1	Pooled growth from 1 biological replicate
2.5% CITMIT-DMDMH-EDTA control	2.5%.1	Pooled growth from 1 biological replicate
10% CITMIT-DMDMH-EDTA	10%	Pooled growth from 1 biological replicate
12.5% CITMIT-DMDMH-EDTA	12.5%.1	Pooled growth from 1 biological replicate
15% CITMIT-DMDMH-EDTA	15%	Pooled growth from 1 biological replicate
Adaptation of biofilm growth to CITMIT-DMDMH-EDTA		
Pooled 1 (TSB planktonic growth replicate 1 & 2)	-	Pooled growth from two populations exposed to the same experimental conditions
Pooled 2 (TSB Bead replicate 1 & 2)	-	Pooled growth from two populations exposed to the same experimental conditions
Pooled 3 (TSB CITMIT-DMDMH-EDTA Bead replicate 1 & 2)	-	Pooled growth from two populations exposed to the same experimental conditions

Footnotes: CITMIT, chloromethylisothiazolinone and methylisothiazolinone blend in the ratio 3:1

Table 2.15 Preservative concentrations used in preservative susceptibility profiling of isolates from preservative adaptation experiments

Preservative concentration (%)		
CITMIT	DMDMH	CITMIT-DMDMH-EDTA
0	0	0
0.000156	0.0078125	2.5
0.00313	0.015625	5
0.000625	0.03125	7.5
0.00125	0.0625	10
0.0025	0.125	12.5
0.005	0.25	17.5
0.01		20
		22.5

Footnotes: CITMIT, chloromethylisothiazolinone and methylisothiazolinone blend in the ratio 3:1; DMDMH, dimethylol dimethyl hydantoin; CITMIT-DMDMH-EDTA, preservative combination where 100% constituted 0.0009% CITMIT, 0.22% DMDMH and 0.1% EDTA dissolved in TSB.

3 Assembly of an industrial isolate collection and evaluation of culture-dependent techniques for the detection of *Pseudomonas aeruginosa*

The comparative study of five selective agars described in this chapter was published within:

Weiser, R., Donoghue, D., Weightman, A. and Mahenthiralingam, E. (2014). Evaluation of five selective media for the detection of *Pseudomonas aeruginosa* using a strain panel from clinical, environmental and industrial sources. *Journal of Microbiological Methods* **99**:8-14.

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3.1 Introduction

Microbial contamination of industrial products is of serious concern as it can lead to product spoilage, product recalls and poses a health risk to vulnerable consumers (Jimenez 2007). Non-sterile consumer products, such as cosmetics and household products, must be manufactured to ensure the microbial burden is below certain threshold levels and no harmful organisms are present (Perry 2001; Sutton and Jimenez 2012). *P. aeruginosa* is one of the most common bacterial species causing contamination of products and is considered an 'objectionable organism' as it is an opportunistic pathogen (Sutton and Jimenez 2012). It is not the only microorganism capable of causing contamination, however, with variety of other Gram-negative bacteria, Gram-positive bacteria, yeasts and moulds being associated with product recall incidents (Jimenez 2007; Sutton and Jimenez 2012). Despite this, few studies have catalogued the bacterial diversity associated with contamination in the home and personal care (HPC) industry. Much of our knowledge is currently derived from reports detailing contamination incidents, such as those generated by the European Commission Rapid Alert System for non-food products posing a serious risk (RAPEX; available at: http://ec.europa.eu/consumers/archive/safety/rapex/) and the U.S Food and Drug Administration (FDA; available at http://www.fda.gov/Safety/Recalls/EnforcementReports/default.htm), or reviews of these publicly available data (Wong *et al.* 2000; Jimenez 2007; Lundov and Zachariae 2008; Sutton and Jimenez 2012). Industrial isolates have therefore largely been overlooked in research and no published industrial strain collections exist. Industrial manufacturers may have their own isolate and contaminated product archives, but these are not generally accessible by research institutions.

The correct identification of industrial contaminants is very important, particularly when a number of common contaminants, including *P. aeruginosa*, are opportunistic pathogens (Sutton and Jimenez 2012). The majority of routine diagnostic tests for *P. aeruginosa*, including those used by industry, are still reliant on culture-dependent methods (Orus and Leranoz 2005; Deschaght *et al.* 2011) which require growth of the target organism. Numerous selective agars are available for the detection of *P. aeruginosa* including those that contain inhibitory selective agents such as antibiotics and biocides (Lilly and Lowbury 1972), those that use positive selection (Szita *et al.* 1998) and those that use chromogenic selection methods (Laine *et al.* 2009). Cetrimide-containing media are commonly used both in clinical and industrial settings (FDA 1998) but can lack selectivity (Kodaka *et al.* 2003; Laine *et al.* 2009). These media are often also designed to enhance pigment production (Brown and Lowbury 1965) as synthesis of pyocyanin can unequivocally identify *P. aeruginosa* (Reyes *et al.* 1981). Only 90-95% of isolates produce pyocyanin (Smirnov and Kiprianova 1990), however, which can confound identification. Media containing acetamide as the positive selective agent have not been evaluated extensively as identification media, although have been reported to have good selectivity and may be useful for detecting low numbers of cells from 'stressed' environments (Szita *et al.* 1998; Szita *et al.* 2007). In these media acetamide represents the sole carbon and nitrogen source, which few species other than *P. aeruginosa* can dissociate to release ammonia and acetic acid (Szita *et al.* 1998). Auxotrophic *P. aeruginosa* isolates, such as those encountered in respiratory infections in cystic fibrosis patients (Thomas *et al.* 2000), have difficulty growing on minimal media meaning that further modification of the acetamide media may be necessary to improve sensitivity. Only one study has evaluated the recently developed chromID® *P. aeruginosa* (manufactured by bioMérieux, La Balme-les-Grottes, France) showing the chromogenic agar to have higher selectivity than Pseudomonas CN selective agar (Oxoid, Basingstoke, UK). By virtue of an unambiguous colour change the medium has demonstrated potential for the simultaneous isolation and identification of *P. aeruginosa* (Laine *et al.* 2009), warranting further assessment.

When attempting to isolate *P. aeruginosa* from a diverse sample, rather than diagnostic identification afforded by growth on selective media, enrichment is a strategy to consider. Enrichment media provide conditions favourable for the growth of a particular organism and ideally discourage the growth of others (Madigan *et al.* 2010). These media have proven useful in detecting low numbers of bacterial cells (Jasson *et al.* 2009) and can aid the recovery of sub-lethally injured cells (Nexmann Jacobsen 1999; Wu 2008), a particular concern for cosmetic microbiologists (Orus and Leranoz 2005). Whilst the incorporation of inhibitory selective agents can prevent the growth of undesirable organisms, it can also compromise the recovery of

injured cells (Nexmann Jacobsen 1999). To circumvent this, an enrichment broth with low inhibitory selective pressure can be used to promote recovery, followed by a selective detection step to isolate the target organism (Jasson *et al.* 2009). Enrichment media which have been used for the isolation of *P. aeruginosa* include acetamide broth, asparagine broth and malachite green broth (Geuenich and Muller 1982). Acetamide broths containing varying concentrations of acetamide (from 2 to 20 g/L) and different overall formulations have been explored to enrich and isolate *P. aeruginosa* from clinical samples (Smith and Dayton 1972; Kelly *et al.* 1983), sewage water (Geuenich and Muller 1982), soil and vegetable samples (Green *et al.* 1974) and water and milk samples (Szita *et al.* 1998; Szita *et al.* 2007). These studies have found that acetamide media facilitate the detection of low numbers of *P. aeruginosa* cells, although may allow the growth of other species with can deaminate acetamide (Oberhofer and Rowen 1974). Potentially, modification of the acetamide concentration and the overall composition of the media could optimise selectivity without inhibiting the growth of *P. aeruginosa* strains.

3.1.1 Aims

The aims of this chapter were as follows:

1) To assemble a collection of industrial isolates (*P. aeruginosa* and non-*P. aeruginosa*) originating from contaminated HPC products and identify these to the species level.

2) To perform a comparison of five selective agars for the detection of *P. aeruginosa* and determine their specificity and sensitivity, using a systematically assembled strain panel of *P. aeruginosa* and non-*P. aeruginosa* strains from diverse sources.

3) To investigate the potential of acetamide media (Z-media) as enrichment media for *P. aeruginosa*, using growth dynamics of *P. aeruginosa* and non-*P. aeruginosa* strains to assess selectivity.

Hypothesis 1: All five selective agars in the comparative study will perform equally well in terms of specificity and sensitivity.

Hypothesis 2: Acetamide media can act as enrichment media for *P. aeruginosa*, permitting the growth of *P. aeruginosa* strains and inhibiting the growth of non-*P. aeruginosa* strains.

3.2 Results

3.2.1 Assembly of an HPC-industrial isolate collection

3.2.1.1 Sourcing of industrial isolates for the collection

The collection was initially established during a 4 week placement at URDPS (April/May 2012); further isolates were added throughout the PhD. Industrial isolates were drawn from both freezer stocks of isolates from previous contamination incidents and also isolated from products held in the product contamination library at URDPS. Both *P. aeruginosa* and non-*P. aeruginosa* isolates from a variety of different home and personal care product (HPC) types were included, in addition to reference strains used in industrial testing. Further isolates and product samples were sent from URDPS to Cardiff after the placement had finished. Overall 105 industrial isolates were obtained from URDPS freezer stocks and 24 different product samples (Table 2.3 and Table 2.4). There was a bias towards *P. aeruginosa* isolates (n=75) as this species was the primary focus of the PhD. As much information as possible was noted about each isolate, including product source and date and location of isolation.

3.2.1.2 Identification of industrial isolates to the species level

Although preliminary identification of many of the industrial isolates had been performed at URDPS, these were verified either by the application of a species-specific PCR or sequencing and analysis of marker genes (Section 2.7). The isolates, their species identities and isolation sources are shown in Table 2.3.

3.2.2 Comparison of five selective agars for the detection of *P. aeruginosa*

To further investigate culture-dependent detection methods for *P. aeruginosa*, a comparative study of five selective agars was performed. Unlike previous studies, which in general have considered four or fewer selective agars with similar selection strategies (Kodaka *et al.* 2003), five selective media were evaluated in parallel and including those using inhibitory selection (PCN and PIA), positive selection (Z-agar, mZ-agar) and chromogenic detection (ChromID). The mZ-agar (acetamide agar) differed from the original Z-agar (Szita *et al.* 1998) by the addition of sufficient nutrient supplementation to facilitate growth of auxotrophic *P. aeruginosa* isolates that are commonly encountered in CF (Thomas *et al.* 2000). The efficacy of these isolation agars across strains, commonly mistaken species and accounting for strain source was considered.

3.2.2.1 Selection of a strain panel

Rather than measuring the performance of these agars as part of prospective surveillance for *P. aeruginosa* in environmental or clinical samples, a bacterial test panel was systematically assembled to include genetically distinct *P. aeruginosa* strains validated by molecular identification and strains from commonly mistaken non-*P. aeruginosa* species (Spilker *et al.* 2004) and Gram-negative pathogens encountered in CF patients (Hauser *et al.* 2011). This was the first media comparison study to include strains originating from clinical, environmental and industrial sources. The strain panel comprised 58 *P. aeruginosa* and 90 non-*P. aeruginosa* strains Table 2.5.

3.2.2.2 Sensitivity of the five media

PCN, PIA and mZ-agar demonstrated 100% sensitivity as all 58 *P. aeruginosa* strains grew on these agars after 24 hours incubation at 37°C (Table 3.1). The sensitivity of the chromogenic agar did not reach this level due to the variable colours produced by certain *P. aeruginosa* strains (Figure 3.1). Overall, 47 out of 58 strains of *P. aeruginosa* strains were correctly identified after 24 hours (81% sensitivity), with 5 strains not displaying adequate growth and 6 giving the wrong colour (yellow, orange, light brown, green and black), which may have reflected a deficiency in β-alanyl aminopeptidase activity. After 72 hours the sensitivity of chromID Pa increased to 95%. Growth on the Z-agars was much weaker than on the other agars (Figure 3.2) and 1 auxotrophic *P. aeruginosa* strain LESB58 (Winstanley *et al.* 2009) did not grow at all on the unmodified Z-agar. The modified Z-agar had nutrient supplementation sufficient for the cultivation of the LESB58 strain although growth was still very weak. There did not appear to be any major differences in growth characteristics between clinical, environmental and industrial strains apart from after 72 hours incubation on chromID Pa, the 3 strains which gave negative results were all from industrial sources. The *P. aeruginosa* panel was also screened for growth on PCN, PIA and chromID Pa at 42°C. Whilst there were no changes in the sensitivity of PCN or PIA at this increased temperature, slight improvements in strain colouration on chromID Pa were observed which led to an increased number of strains recorded as positive: after 24 and 72 hours the sensitivity of chromID Pa was 81% and 95%, respectively.

Table 3.1 Growth of *Pseudomonas aeruginosa* strains on selective media at 37°C

Species	Source	Nº Strains	Nº strains exhibiting growth									
			PCN[a]		PIA[b]		chromID Pa[c]		Z-agar		mZ-agar[d]	
			24 hours	72 hours	24 hours	72 hours	24 hours	72 hours	24 hours	72 hours	24 hours	72 hours
P. aeruginosa	CLIN	22	22	22	22	22	18	22	21	21	22	22
	ENV	6	6	6	6	6	5	6	6	6	6	6
	IND	30	30	30	30	30	24	27	30	30	30	30
Total		58	58	58	58	58	47	55	57 (3+/-)[e]	57 (1+/-)	58 (2+/-)	58 (1+/-)
Sensitivity (%)			100	100	100	100	81	95	98	98	100	100

[a] PCN, Pseudomonas CN selective agar; [b] PIA, Pseudomonas isolation agar; [c] chromID Pa, chromID® *P. aeruginosa*; a positive result on chromID Pa was taken as growth accompanied by a red/pink/pink-brown/purple colouration; [d] mZ-agar, modified Z-agar; [e]Weakly growing strains (+/-) were recorded as an overall positive but are highlighted in the tables; CLIN, clinical; ENV, environmental; IND, industrial

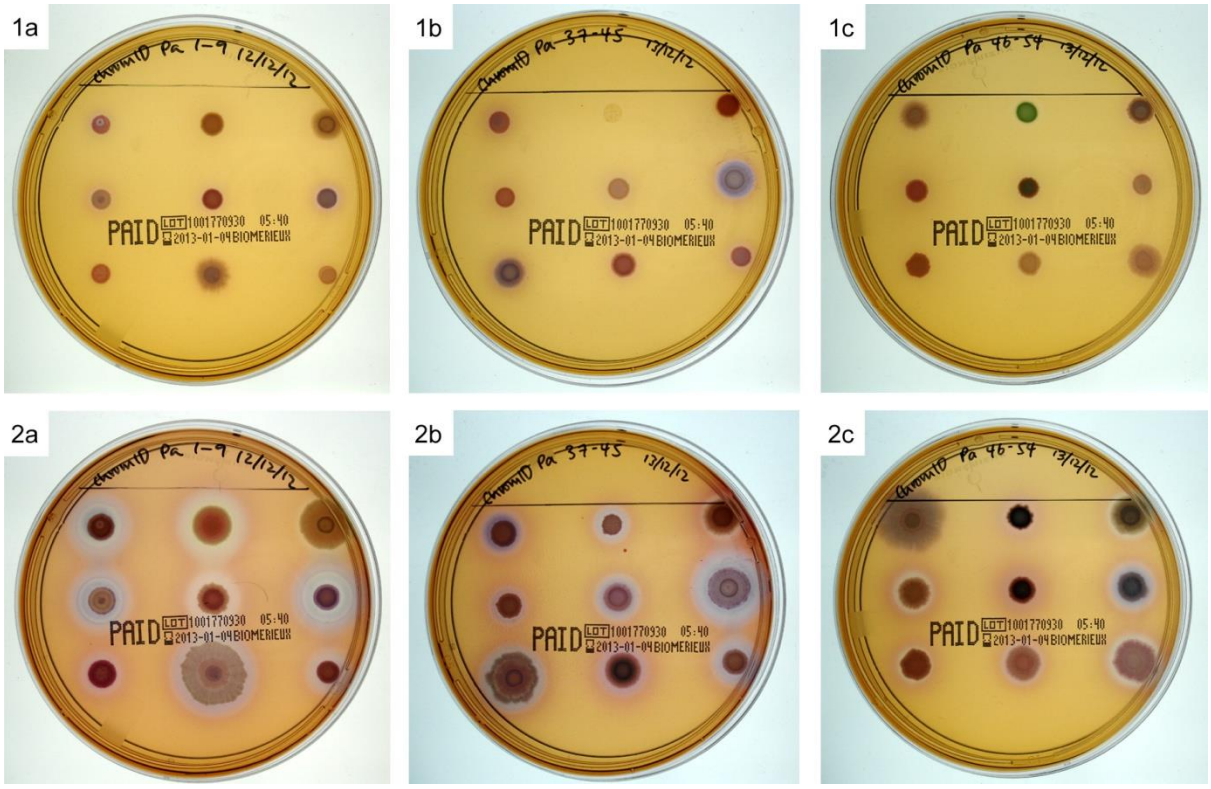


Figure 3.1 Variable colony morphology of *Pseudomonas aeruginosa* strains on chromID® *P. aeruginosa*. Results for 27 *P. aeruginosa* strains are shown after 24 hours (1a, 1b and 1c) and 72 hours (2a, 2b and 2c) incubation at 37°C. Growth accompanied by a red, pink, pink-brown or purple colour was recorded as a positive result for *P. aeruginosa*. Strains grew to produce a range of other colours including yellow (1a top row right), orange (1a top row middle), brown (not shown in photos), green (1c top row middle) and black (1c middle row middle). The sensitivity of chromID® *P. aeruginosa* increased after 72 hours incubation.

3.2.2.3 Pigment production

Pigment production on TSA, PCN and PIA was variable (Table 3.2). On TSA pyocyanin production was easily identified but as it was often difficult to distinguish between pyocyanin and pyoverdin on PCN and PIA, strains were scored as positive or negative for production of either/both pigments (Figure 3.2). Over 90% of strains produced pigment on all three agars after 24 hours incubation. PIA was the most successful at enhancing pigment production; 100% of strains were observed to produce pigment after 72 hours incubation. The majority of strains produced a light green/yellow pigment on PIA indicative of pyoverdin rather than pyocyanin production.

Table 3.2 Pigment production by *Pseudomonas aeruginosa* strains at 37°C

Selective agar	**No strains producing pigment[a] (%)**	
	24 hours	**72 hours**
Tryptone Soya Agar	53/58 (91)	56/58 (97)
Pseudomonas CN selective agar	41/58 (71)	57/58 (98)
Pseudomonas isolation agar	47/58 (81)	58/58 (100)

[a]Strains producing pyocyanin, pyoverdin or both pigments

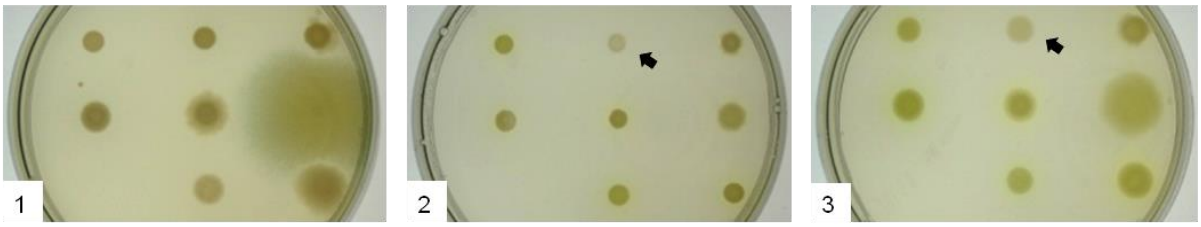


Figure 3.2 Pigment production by *Pseudomonas aeruginosa* strains.Panels show growth of 8 *P. aeruginosa* strains after 24 hours incubation at 37°C on (1) Tryptone soya agar (TSA), (2) Pseudomonas CN selective agar (PCN) and, (3) Pseudomonas isolation agar (PIA). Black arrows indicate a strain negative for pigment production on PCN and PIA.

3.2.2.4 Selectivity of the five media

The most selective of the 5 media was chromID Pa (Table 3.3), which after 72 hours incubation at 37°C had a specificity of 70%. Strains of *Burkholderia* (n=15), other *Pseudomonas* (n=12) and *Ochrobactrum* (n=1) species exhibited growth on chromID Pa that had a similar colouration to strains of *P. aeruginosa*. Although at 24 hours Z-agar had the second highest specificity of 42%, this dropped to 26% after 72 hours. It was also very difficult to determine if true growth had occurred on both of the Z-agars (Figure 3.3) which resulted in a high proportion of positive results being recorded as weak (+/-) rather than true positive. The additional nutrient supplementation in the modified Z-agar led to an increase in non-*P. aeruginosa* strains that could grow, and overall this agar was the least selective of the 5, having a specificity of 6% after 72 hours incubation. The two agars containing inhibitory selective agents, PCN and PIA, performed very similarly having specificities of 30% and 23%, respectively, after 72 hours incubation. In general, strains of *Achromobacter* (n=2), *Burkholderia* (n=28) and *Pseudomonas* (PIA n=23; PCN n=22) species were most likely to give false positives on PCN and PIA.

Incubation at 42°C for 72 hours increased the specificities of PCN and PIA to 61% and 47%, respectively. These incubation conditions, however, also elevated the number of false positives recorded for chromID Pa, which decreased its specificity from 80% to 69% at 24 hours and 70% to 68% at 72 hours. Of the 28 strains positive for growth at 37°C on chromID after 24 hours incubation, 9 of these could not grow at 42°C, but 19 'new' false positives were observed. The effect of increased temperature on the selectivity of Z-agar and modified Z-agar was not investigated due to the weak growth already seen at 37°C. Finally, it was observed that strains from different sources grew equally well on the 5 media tested in this study.

Table 3.3 Growth of non-*Pseudomonas aeruginosa* strains on selective media at 37°C

Species	Source	Nº Strains	Nº strains exhibiting growth									
			PCN[a]		PIA[b]		ChromID Pa[c]		Z-agar		mZ-agar[d]	
			24 hours	72 hours	24 hours	72 hours	24 hours	72 hours	24 hours	72 hours	24 hours	72 hours
Achromobacter xylosoxidans	CLIN	2	2	2	2	2	0	0	1 (1 +/-)	2	2 (1 +/-)	2
Acinetobacter species	IND	1	0	0	0	0	0	0	1 (1 +/-)	1	1 (1 +/-)	1
Bacillus species	IND	1	0	0	0	0	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Burkholderia ambifaria	CLIN	1	1	1	1	1	0	0	0	0	0	1 (1 +/-)
	ENV	2	2 (1 +/-)	2	2	2	1	1	0	1 (1 +/-)	1 (1 +/-)	2 (1 +/-)
Burkholderia cenocepacia	CLIN	7	6	6	7	7	2	6	5 (5 +/-)	6 (6 +/-)	3 (3 +/-)	7 (6 +/-)
	ENV	2	2	2	2	2	0	1	0	2	0	2 (2 +/-)
	IND	1	0	1	1	1	1	1	1	1	1	1
Burkholderia cepacia	CLIN	1	1	1	1	1	0	0	1	1	1	1
Burkholderia cepacia complex	IND	2	1	2 (1+/-)	1	1	1	1	2 (1 +/-)	2	2	2
Burkholderia contaminans	CLIN	1	1 (1 +/-)	1	1	1	0	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Burkholderia dolosa	CLIN	1	1	1	1	1	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Burkholderia gladioli	CLIN	1	0	0	0	0	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Burkholderia lata	ENV	1	1	1	1	1	1	1	1	1	1	1
Burkholderia multivorans	CLIN	4	4	4	4	4	2	2	3 (3 +/-)	3 (3 +/-)	3 (3 +/-)	4 (4 +/-)
	ENV	1	1	1	1	1	1	1	0	0	1 (1 +/-)	1 (1 +/-)
Burkholderia phymatum	ENV	1	0	0	0	0	0	0	0	0	0	1 (1 +/-)
Burkholderia phytofirmans	ENV	1	0	0	0	0	0	0	0	0	0	1 (1 +/-)
Burkholderia pyrrocinia	CLIN	1	1	1	1	1	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Burkholderia stabilis	CLIN	1	1 (1 +/-)	1	1	1	1	1	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Burkholderia vietnamiensis	CLIN	1	1	1	1	1	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
	ENV	1	1 (1 +/-)	1	1	1	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
	IND	1	1 (1 +/-)	1	1	1	0	0	1 (1 +/-)	1	1 (1 +/-)	1
Escherichia coli	CLIN	1	0	0	0	0	0	0	0	0	1 (1 +/-)	1 (1 +/-)
Halomonas species	IND	1	0	0	0	1	0	0	0	1 (1 +/-)	1 (1 +/-)	1
Ochrobactrum anthropi	IND	1	0	0	0	0	1	1	1 (1 +/-)	1	1 (1 +/-)	1
Pandoraea apsita	CLIN	2	2	2	2	2	0	0	2 (2 +/-)	2 (1 +/-)	2 (2 +/-)	2 (1 +/-)
Pandoraea pnomenusa	CLIN	3	3 (3 +/-)	3 (3 +/-)	3	3	0	0	3 (3 +/-)	3	3 (2 +/-)	3
Pandoraea pulmonicola	CLIN	2	2	2	2	2	0	0	2 (2 +/-)	2 (1 +/-)	2 (2 +/-)	2 (1 +/-)
Pandoraea sputorum	CLIN	4	3	3	4	4	0	0	4 (4 +/-)	4 (1 +/-)	4 (4 +/-)	4 (1 +/-)
Pantoea agglomerans	IND	1	0	0	0	0	0	0	0	0	1 (1 +/-)	1 (1 +/-)
Pseudomonas alcaligenes	ENV	1	1	1	0	1	0	0	0	0	1 (1 +/-)	1 (1 +/-)
Pseudomonas aureofaciens	ENV	1	1	1	1	1	1	1	0	0	1 (1 +/-)	1 (1 +/-)
Pseudomonas fluorescens	ENV	1	0	0	1 (1 +/-)	1 (1+/-)	0	0	0	0	0	0
	IND	2	2	2	2	2	0	2	0	0	0	0
Pseudomonas mendocina	ENV	1	1	1	1 (1 +/-)	1	1	1	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)

Pseudomonas oleovorans	IND	1	1 (1 +/-)	1	0	0	0	0	1 (1 +/-)	1	1 (1 +/-)	1
Pseudomonas pseudoalcaligenes	IND	1	1 (1 +/-)	1	0	1	0	0	0	1 (1 +/-)	1 (1 +/-)	1
Pseudomonas putida	ENV	1	1 (1 +/-)	1	1	1	0	0	0	0	0	0
	IND	7	6 (1 +/-)	7 (1 +/-)	7	7	3	4	6 (6 +/-)	6 (3 +/-)	7 (7 +/-)	7 (4 +/-)
Pseudomonas species	IND	8	7 (1 +/-)	8 (2 +/-)	6	7	2	3	1 (1 +/-)	5 (5 +/-)	6 (6 +/-)	7 (6 +/-)
Ralstonia insidiosa	CLIN	1	0	0	0	0	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Ralstonia mannitolytica	CLIN	2	0	0	0	0	0	0	2 (2 +/-)	2 (2 +/-)	2 (2 +/-)	2 (2 +/-)
Ralstonia pickettii	CLIN	3	0	0	0	0	0	0	3 (3 +/-)	3 (3 +/-)	3 (3 +/-)	3 (1 +/-)
Serraria marcescens	ENV	2	0	0	2	2	0	0	-	-	-	-
	IND	1	0	0	1	1	0	0	-	-	-	-
	NA	1	0	0	1	1	0	0	-	-	-	-
Staphylococcus aureus	CLIN	2	0	0	0	0	0	0	0	0	1 (1 +/-)	2 (2 +/-)
Staphylococcus epidermidis	CLIN	1	0	0	0	0	0	0	0	0	1 (1 +/-)	1 (1 +/-)
Stenotrophomonas maltophila	CLIN	1	0	0	0	1	0	0	0	1 (1 +/-)	1 (1 +/-)	1 (1 +/-)
Stenotrophomonas species	IND	1	0	0	1	1	0	0	0	1 (1 +/-)	1 (1 +/-)	1
Total			59/90 (13 +/-)[e]	63/90 (7 +/-)	65/90 (2 +/-)	69/90 (1 +/-)	18/90	27/90	50/86 (46 +/-)	64/86 (39 +/-)	68/86 (61 +/-)	81/86 (51 +/-)
Specificity (%)			34	30	28	23	80	70	42	28	26	6

[a] PCN, Pseudomonas CN selective agar
[b] PIA, Pseudomonas isolation agar
[c] chromID Pa, chromID® *P. aeruginosa*; a positive result on chromID Pa was taken as growth accompanied by a red/pink/pink-brown/purple colouration
[d] mZ-agar, modified Z-agar
[e] Weakly growing strains (+/-) were recorded as an overall positive but are highlighted in the tables
CLIN, clinical; ENV, environmental; IND, industrial; NA, source unknown

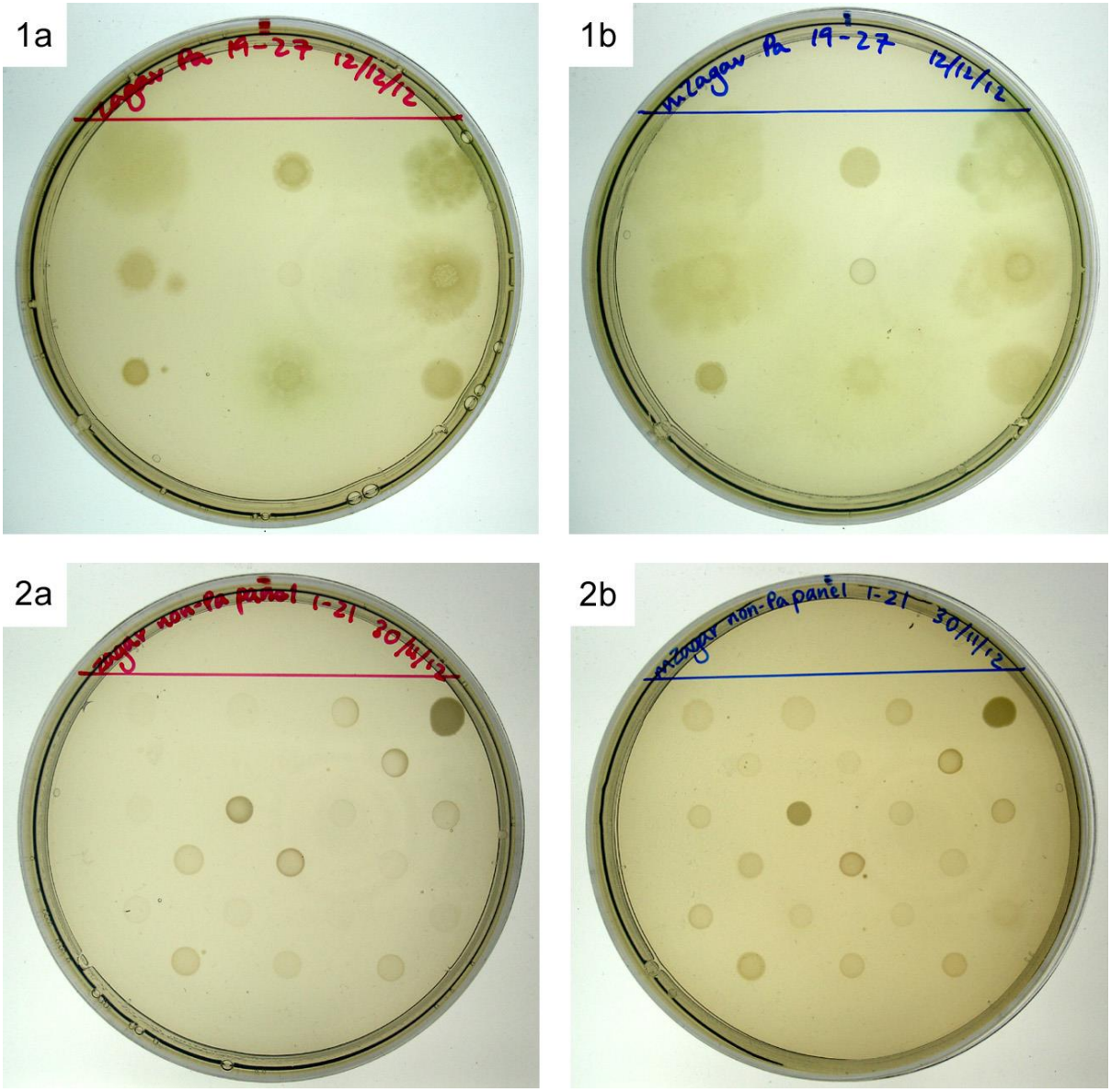


Figure 3.3 Growth of *Pseudomonas aeruginosa* and non-*P. aeruginosa* strains on Z-agar and modified Z-agar. Results are shown for 9 *P. aeruginosa* strains (1a and 1b) and 21 non-*P. aeruginosa* strains (2a and 2b) after 72 hours incubation at 37°C. Z-agar plates are marked with red and are shown in panels 1a and 2a. Modified Z-agar plates are marked with blue and shown in panels 1b and 2b. Growth was weak for both *P. aeruginosa* and non-*P. aeruginosa* strains on the Z-agars and it was difficult to determine whether true growth had occurred in many cases (for example 1a middle row middle). The addition of extra nutrient supplementation in the modified Z-agar encouraged the growth of *P. aeruginosa* strains (compare 1a to 1b) but also led to an increase in the number of non-*P. aeruginosa* strains that could grow on the agar (compare 2a to 2b).

3.2.2.5 Presumptive identification of *P. aeruginosa*

The PPV and NPV values for the agars at 37°C and 42°C are given in Table 3.4. For this strain panel chromID Pa had the highest PPV at 37°C (72% and 67% at 24 and 72 hours, respectively) and at 42°C after incubation for 72 hours (62%). Due to the increased numbers of chromID Pa false positives at 42°C, PCN was the agar with the highest PPV at this increased temperature after 24 hours incubation (68%). ChromID Pa and Z-agar were the only agars with a NPV less than 100% as not all *P. aeruginosa* strains were correctly identified on these media. The lowest NPV was for chromID after 24 hours incubation at 37°C (87%), although this increased to 95% after 72 hours incubation and 98% at 42°C regardless of incubation duration.

Table 3.4 Positive and negative predictive values for the selective agars

Agar	PPV (%)				NPV (%)			
	37°C		42°C		37°C		42°C	
	24 hrs	72 hrs	24 hrs	72 hrs	24 hrs	72 hrs	24 hrs	72 hrs
PCN	50	48	68	62	100	100	100	100
PIA	47	46	62	55	100	100	100	100
chromID Pa	72	67	67	67	87	95	98	98
Zagar	53	47			97	96		
mZagar	46	42			100	100		

PPV, Positive predictive value; NPV, negative predictive value
PCN, Pseudomonas CN selective agar; PIA, Pseudomonas isolation agar; chromID Pa, chromID™ *P. aeruginosa*; mZ-agar, modified Z-agar

3.2.3 Acetamide media as enrichment media for *P. aeruginosa*

To investigate the suitability of acetamide media as enrichment media for *P. aeruginosa*, the growth dynamics of *P. aeruginosa* and non-*P. aeruginosa* strains in Z-broth (Szita *et al.* 1998) containing different concentrations of acetamide were examined. Data detailing 72 hours growth in acetamide broths was generated using the Bioscreen C instrument and analysed using a combination of Microsoft Excel, the groFit package for R and Boxplot R. Of particular interest was whether increasing the concentration of acetamide would inhibit the growth of non-*P. aeruginosa* strains, whilst permitting, or even encouraging, the growth of *P. aeruginosa* strains.

3.2.3.1 Effect of increasing the acetamide concentration: growth in Z-broth containing up to 20 g/L acetamide

The growth curves of the *P. aeruginosa* strains PAO1, RW109, RW110, LESB58 and RW113 and the non-*P. aeruginosa* strains *P. putida* RW112, *B. lata* 383 and *B. dolosa* LMG 18943 in the presence of 1, 4, 10 and 20 g/L acetamide are shown in Figure 3.4. The strains split into three broad groups: 1) those demonstrating 'typical' growth curves, exiting exponential phase at approximately 20-24 hours (*P. aeruginosa* PAO1, RW109 and RW110), 2) those with 'atypical' growth curves lacking a well-defined exponential phase and whose OD increased gradually, reaching their maximum after 40 hours (*P. aeruginosa* RW113 and *B. lata* 383), and 3) those whose OD never reached above 0.03 (*P. aeruginosa* LESB58, *P. putida* RW112 and *B. dolosa* LMG 18943) (Figure 3.4).

To more comprehensively model the growth curve parameters (length of lag phase, maximum growth rate and maximum culture density reached) the groFit package for R statistical software was used. The *P. aeruginosa* strains LESB58 and RW113 and the non-*P. aeruginosa* strains *P. putida* RW112, *B. lata* 383 and *B. dolosa* LMG 18943 were excluded from the complete analysis as they produced 'atypical' growth curves that could not be modelled accurately by groFit, possibly because of their long lag phase and/or poorly defined exponential phase. The growth parameters lag, phase, growth rate and maximum culture density reached are illustrated for the three prototrophic *P. aeruginosa* strains in Figure 3.5. Only the maximum OD reached (spline fit data), taken directly from the OD measurements, were analysed for the other *P. aeruginosa* and non-*P. aeruginosa* strains. These data are shown alongside the maximum OD data for the prototrophic *P. aeruginosa* strains in Figure 3.6. Although responses were strain specific, in general, increasing the acetamide concentration to 20 g/L did not inhibit the growth of prototrophic strains to below the levels seen at 1 g/L acetamide, with the exception of increasing the length of the lag phase for PAO1 and RW109. In addition the maximum ODs

reached for PAO1, RW109 and RW110 were determined to be significantly increased at 20 g/L compared to 1 g/L acetamide ($p<0.05$; Welch 2-sample t-test performed in R statistical software). There was very little change in the maximum OD reached for *P. aeruginosa* LESB58, *P. putida* RW112 or *B. dolosa* LMG 18943. There was, however, a slight increase in the maximum OD obtained for the weakly growing *P. aeruginosa* strain RW113, suggesting that elevated concentration of acetamide actually encouraged the growth of this strain. The largest effect was observed for *B. lata* 383, whose maximum OD dropped from 0.38 at 4 g/L to 0.07 at 20 g/L acetamide.

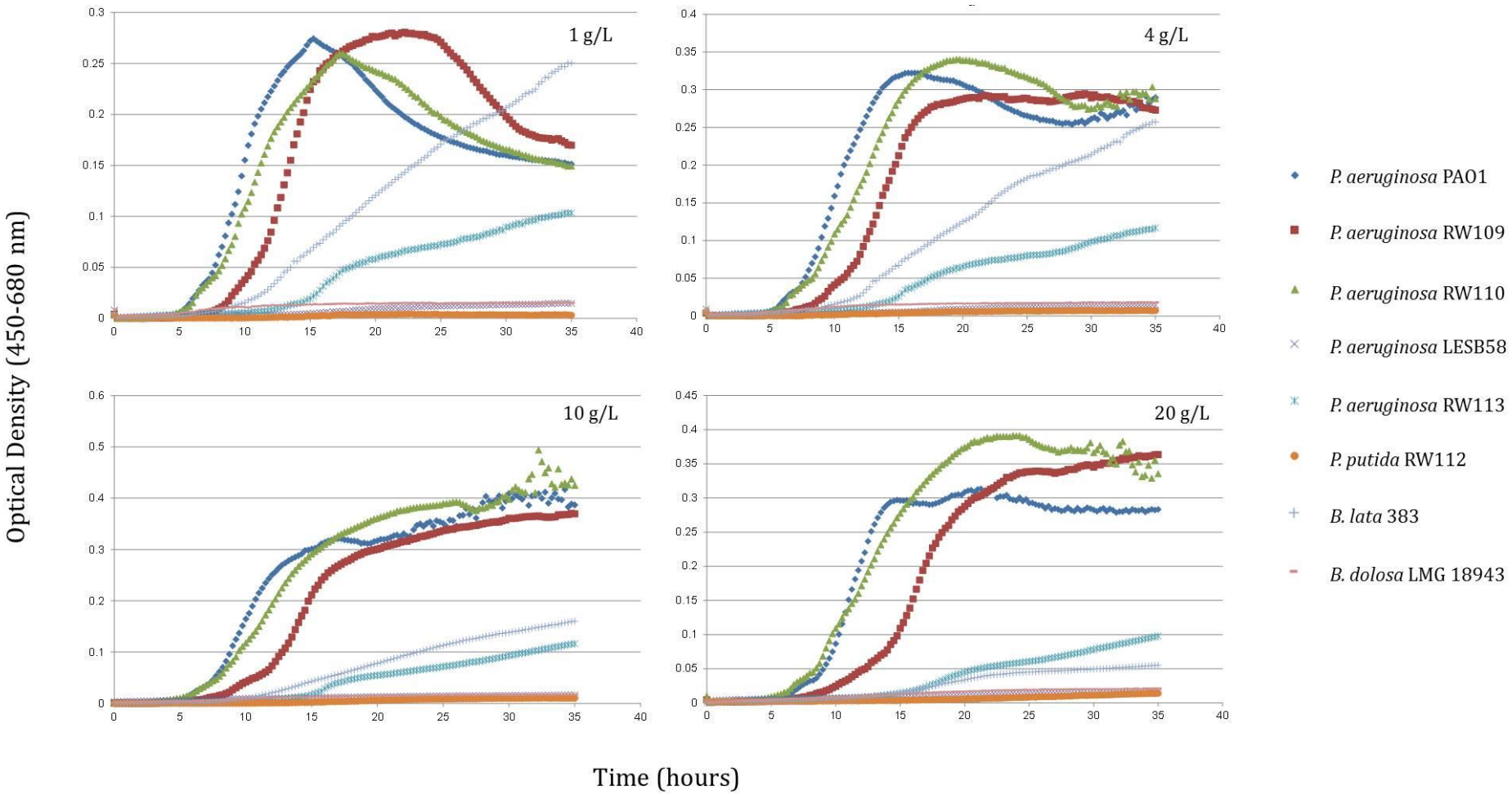


Figure 3.4 Effect of acetamide concentrations on bacterial growth. Growth curves of *P. aeruginosa* and non-*P. aeruginosa* strains in Z-broth containing 1, 4, 10 and 20 g/L acetamide over 35 hours are shown.

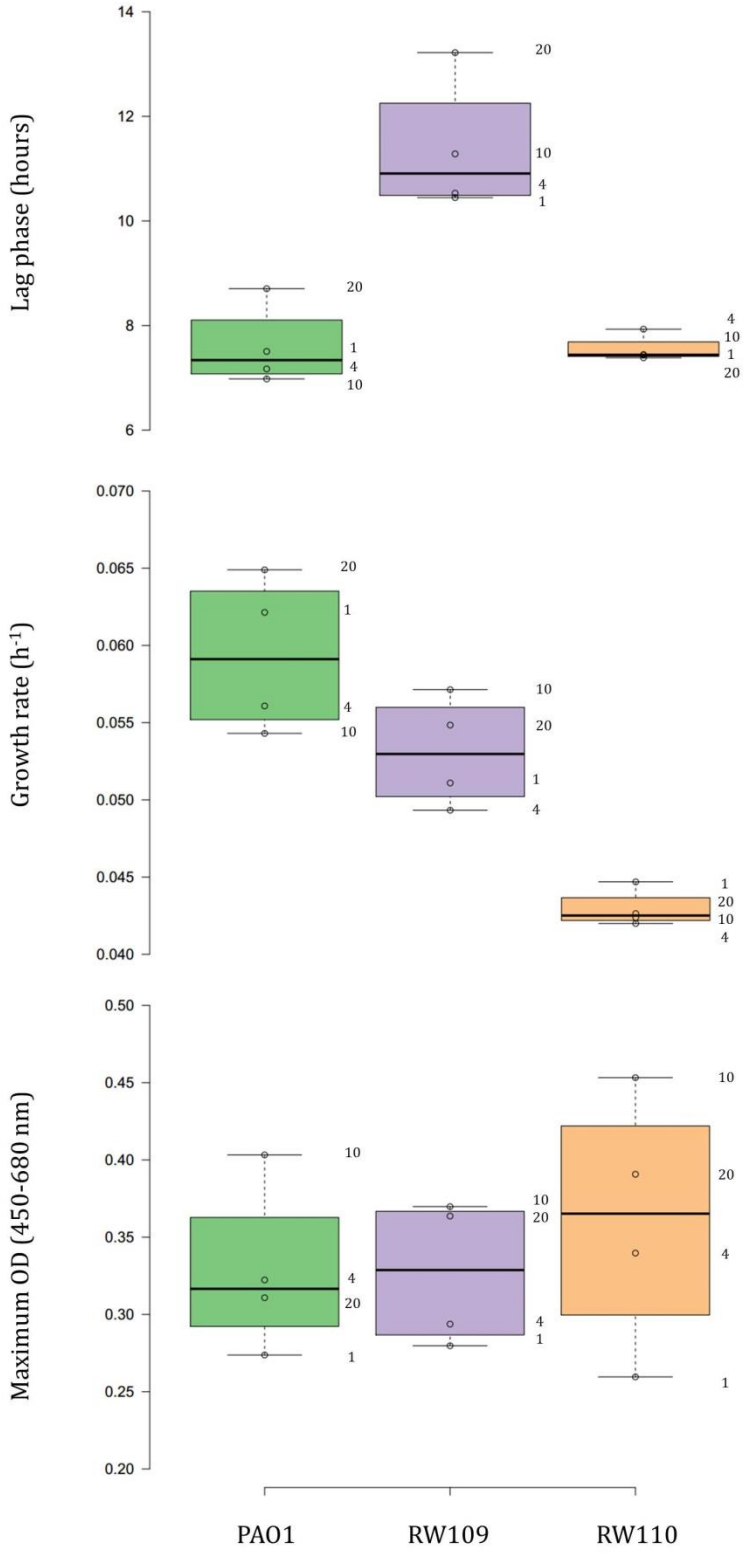


Figure 3.5 Effect of acetamide concentration on length of lag phase, growth rate and maximum culture density reached for *P. aeruginosa* strains. The growth parameters of the *P. aeruginosa* strains PAO1, RW109 and RW110 for growth in Z-broth containing 1, 4, 10 or 20 g/L acetamide over 72 hours are shown. Data points are displayed as open circles and the corresponding concentrations (g/L) of acetamide in the Z-broth are indicated directly to the right of each boxplot.

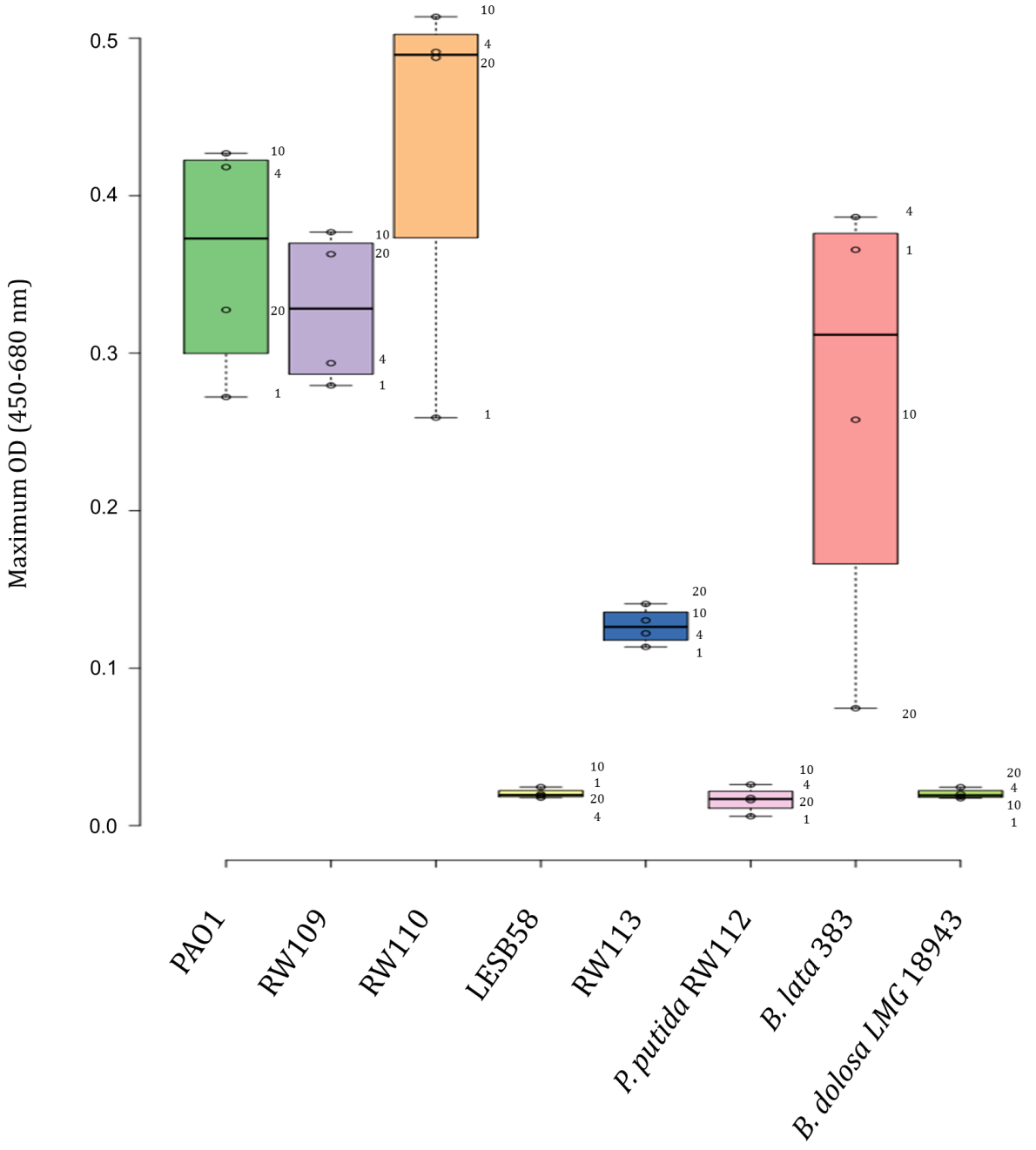


Figure 3.6 Effect of acetamide concentration on maximum culture density. The maximum culture densities reached at different acetamide concentrations for *P. aeruginosa* (PAO1, RW109, RW110, LESB58 and RW113) and non-*P. aeruginosa* strains in Z-broth over 72 hours are shown. Data points are displayed as open ovals and the corresponding concentrations (g/L) of acetamide in the Z-broth are indicated directly to the right of each boxplot.

3.2.3.2 Effect of elevated acetamide concentration: growth in Z-broth containing up to 50 g/L acetamide

As increasing acetamide concentrations up to 20 g/L did not have an inhibitory effect on prototrophic, auxotrophic or weakly growing *P. aeruginosa* strains, did not enhance the growth of *P. putida* RW112 or *B. dolosa* LMG 18943, and considerably reduced the maximum OD of *B. lata* 383, the consequences of increasing the acetamide concentration further were investigated. One prototrophic *P. aeruginosa* strain (PAO1), the weakly growing *P. aeruginosa* strain (RW113) and *B. lata* 383 were grown in Z-broth containing 4, 20, 30, 40 and 50 g/L acetamide (Figure 3.7) and growth dynamics monitored over 72 hours. Using groFit for R statistical software, growth curve parameters could only be estimated for *P. aeruginosa* PAO1 at 20, 30 and 40 g/L acetamide, and for *P. aeruginosa* RW113 at 4 g/L acetamide. The maximum OD (spline fit) data, however, were used in analysis to investigate culture density reached over 72 hours for all three strains (Figure 3.8).

Increasing the acetamide concentration from 20 g/L to 30 g/L inhibited the growth of *P. aeruginosa* PAO1 strain, increasing the lag phase from 7.7 to 17.8 hours, decreasing the growth rate from 0.04 to 0.02 h^{-1} and decreasing the maximum culture density from 0.52 to 0.33. The maximum OD continued to decrease when the acetamide concentration increased, with a value of less than at 0.02 50 g/L. Increasing the concentration above 30 g/L decreased the maximum OD for all three strains (figure 3.8), and overall 20 and 30 g/L acetamide achieved the highest OD values for *P. aeruginosa* PAO1 and RW113 respectively. The greatest inhibitory effect on *B. lata* 383 was exerted between 4 and 20 g/L acetamide, demonstrated by a decrease in maximum OD from 0.26 to 0.013. Above 20 g/L there was a much smaller decrease in the maximum OD of *B. lata* 383, to 0.05 at 50 g/L.

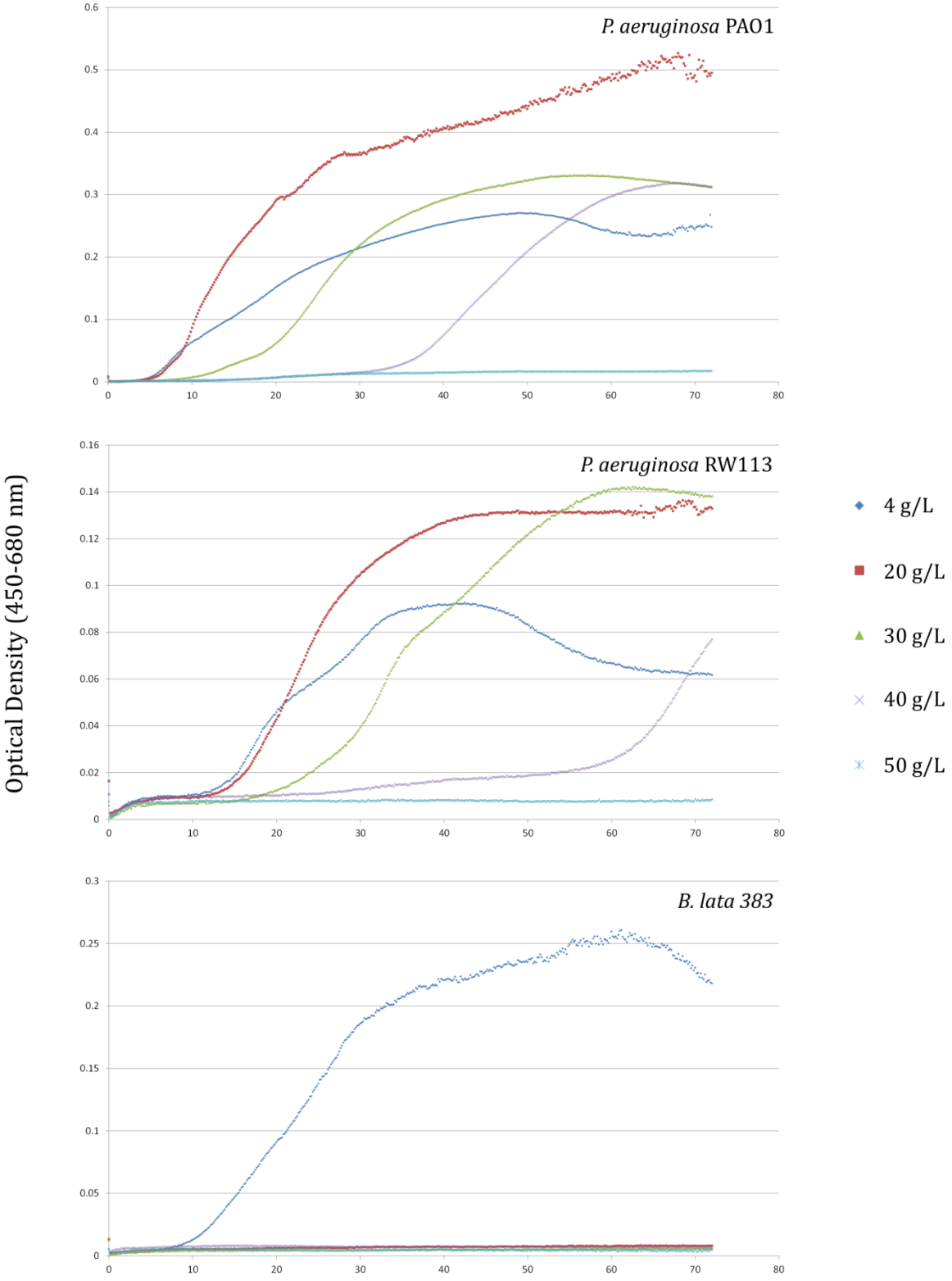


Figure 3.7 Effect of elevated acetamide concentrations on selected strains. The growth curves of *P. aeruginosa* PAO1 and RW113 and *B. lata* 383 Z-broth containing 4, 20, 30, 40 and 50 g/L acetamide over 72 hours are shown.

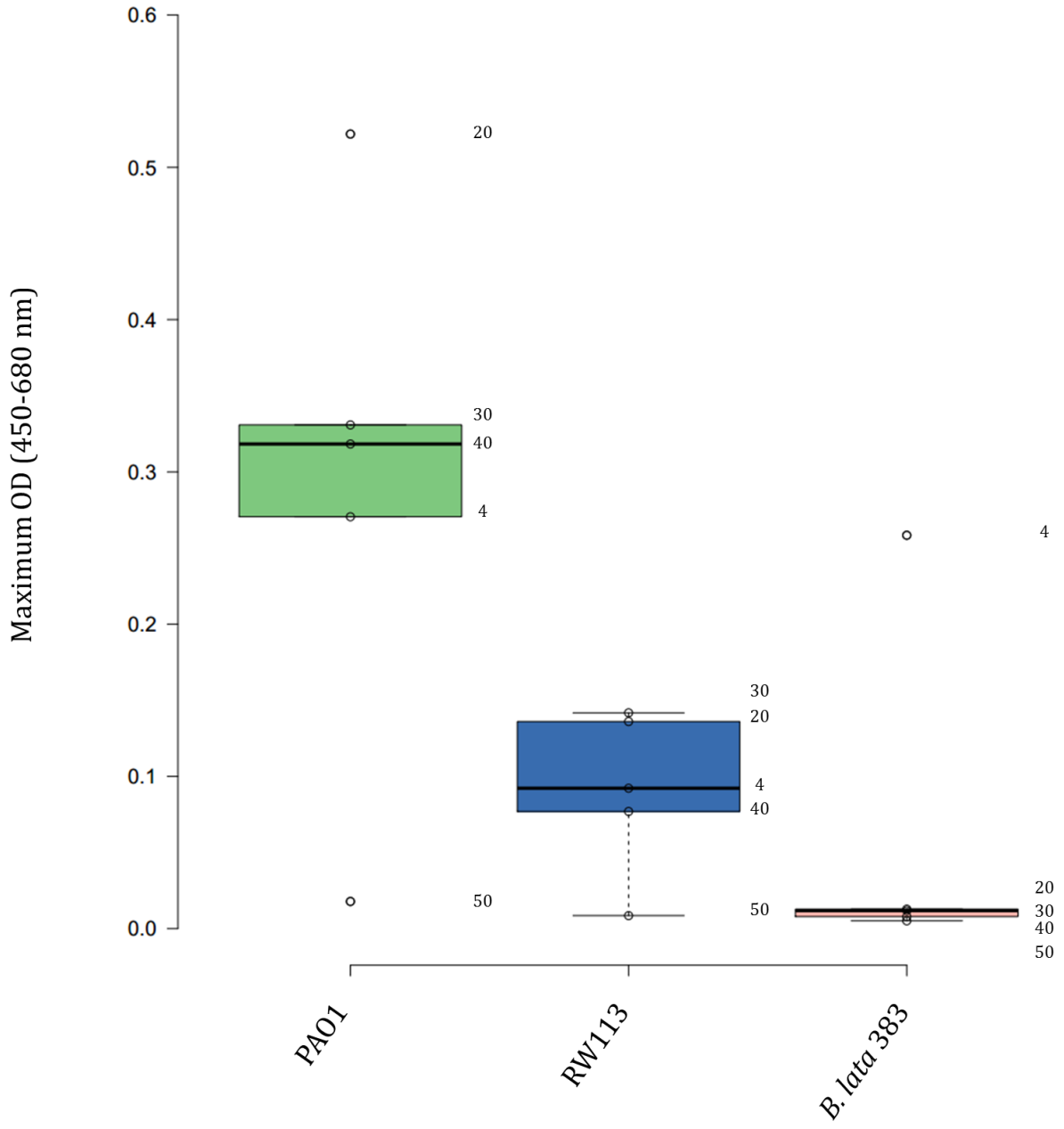


Figure 3.8 Effect of elevated acetamide concentrations on maximum culture density. The maximum culture densities at different acetamide concentrations for *P. aeruginosa* PAO1 and Pa113 and *B. lata* 383 in Z-broth over 72 hours. Data points are displayed as open circles and the corresponding concentrations (g/L) of acetamide in the Z-broth are indicated directly to the right of each boxplot.

3.3 Discussion

A large collection of isolates contaminating HPC products was assembled and identified to the species level using a combination of species-specific PCRs and the sequencing and analysis of marker genes. Marker genes other than the 16S rRNA gene were chosen to identify certain isolates within the collection as the 16S rRNA gene lacks resolution for some species (Janda and Abbott 2007). In the case of suspected *Pseudomonas* and *Burkholderia* species a combination of 16S rRNA gene and *rpoD* (Mulet *et al.* 2010) or *recA*/*gyrB* (Spilker *et al.* 2009) gene sequencing and analysis was used, as these genes have been found to be more discriminatory. In many cases it was not possible to obtain a species ID for isolates of the genus *Pseudomonas*, even with the use of the *rpoD* gene sequence; these isolates were deposited in the collection as *Pseudomonas* spp. Although the collection was biased towards *P. aeruginosa* isolates as the study focus organism, other contaminating species were sampled to populate the collection for culture-dependent and –independent studies. These were chosen to include species that are commonly misidentified as *P. aeruginosa* by culture-dependent methods, such as other *Pseudomonas* species. Whilst the majority of the non-*P. aeruginosa* isolates in the collection were also considered common contaminants of HPC products, *Pseudomonas* and *Burkholderia* species in particular, (Sutton and Jimenez 2012), they did not represent the entire range of different species encountered in industry. Using this collection, a comparative study of five selective agars for the detection of *P. aeruginosa* was performed and additional investigations into acetamide media as enrichment broths for *P. aeruginosa* were carried out.

3.3.1 Comparison of five selective agars for the detection of *P. aeruginosa*

There have been many selective media developed for the isolation of *P. aeruginosa* from clinical and environmental samples. The majority of media currently available for the isolation of *P. aeruginosa* were developed some decades ago and routinely used media, such as those containing cetrimide, lack selectivity (Kodaka *et al.* 2003). In addition, even though the majority of diagnostics are still based on culture-dependent methods, few novel media have been recently developed and few studies have systematically evaluated the performance of available media.

The strain collection used in this comparative study was drawn from clinical and environmental sources, in addition to including strains from industrial contamination incidents. It was the first study to include industrial strains, which represented just under half of the *P. aeruginosa* and approximately a third of the non-*P. aeruginosa* sub-panels. No major differences were observed, however, in the growth characteristics of the industrial strains compared to the rest of the

panel. The choice of panel strains perhaps reflected the low levels of specificity and PPV values observed with the PCN and PIA agars as strains of *Achromobacter*, *Burkholderia* and *Pseudomonas* species are known to have broad antimicrobial resistance (Hogardt *et al.* 2009; Li and Nikaido 2009; Rose *et al.* 2009; Bador *et al.* 2011), and have previously been shown to grow on PCN (Kodaka *et al.* 2003), although no studies have evaluated PIA. PCN and PIA are currently widely available commercially and performed very similarly both in terms of sensitivity and specificity. Both are also formulated to encourage pigment production, being based on King's A medium (King *et al.* 1954).

Although on TSA the primary pigment being produced appeared to be pyocyanin, with 97% of strains being pigmented, growth on PCN and PIA was largely accompanied by the production of a light green/yellow pigment more indicative of pyoverdin. This pigment alone cannot positively identify *P. aeruginosa* as all other fluorescent pseudomonads are capable of pyoverdin production (Leisinger and Margraff 1979). In addition, non-pigmented strains of *P. aeruginosa* may resemble other species that can grow on PCN and PIA. This highlights that although pigment production can guide identification, subsequent diagnostic tests are required to obtain conclusive results. In clinical and industrial settings, growth at 42°C is often used to differentiate *P. aeruginosa* from other bacterial species, although this growth characteristic is not exclusive to *P. aeruginosa*. Incubation at 42°C was considered in the current study and was found to increase the specificity and PPV of PCN and PIA. A variety of chromogenic media have been designed for the detection of pathogens including *P. aeruginosa*. Through inclusion of chromogenic substrates for an enzyme produced by the target species, chromogenic media aim to simplify the isolation and identification process by linking an unambiguous colour change with growth of a target organism (Perry and Freydière 2007). chromID® *P. aeruginosa* was developed by bioMérieux for the detection of *P. aeruginosa* and has been evaluated by one study (Laine *et al.* 2009) which found the medium to have higher selectivity than PCN. The present study also determined chromID Pa to have superior selectivity but the lowest overall sensitivity. If results for chromID Pa were read after 24 hours incubation at 37°C, as suggested by the manufacturer, there was a considerable risk of false negatives, although after 72 hours incubation the sensitivity was greatly improved. This corroborates what was found by (Laine *et al.* 2009) who observed the sensitivity to be only 58% after 24 hours, increasing to 95% after 72 hours.

One of the main issues with chromID Pa was the interpretation of the colony colourings, which were very variable. The spectrum of colours considered a positive result was expanded in this study to include pink-brown as at 37°C this increased the sensitivity (11% increase at 24 hours

incubation) without greatly compromising specificity (3% decrease at 24 hours incubation). Interpretation of colony colouration needs to be re-considered in future studies to ensure that maximum sensitivity is achieved. At the higher incubation temperature of 42°C more *P. aeruginosa* strains grew producing a colour considered positive. Although the change was only slight in most cases, from a brown to a pink-brown colour, this led to an increase in the sensitivity of the medium (81% and 95% after 24 and 72 hours, respectively). A decrease in the specificity and PPV of the medium was also observed, however, as more non-*P. aeruginosa* strains recorded as false positives. Overall, the results for this medium were more reliable when an incubation temperature of 37°C was applied.

Media containing acetamide as the positive selective agent (Z-agar/Z-broth) have been reported to have specificity comparable to that of nitrofurantoin broth (Szita *et al.* 1998), although their sensitivity has not been evaluated. The modification of Z-agar to include additional nutrient supplementation permitted the growth of all of the *P. aeruginosa* strains, including one auxotrophic CF strain. This modification, however, also decreased the selectivity of the agar resulting in mZ-agar having the lowest specificity in the study. After 72 hours the specificity of the unmodified Z-agar was also relatively low and growth of *P. aeruginosa* and non-*P. aeruginosa* strains was weak on both acetamide agars making interpretation of results very difficult. In addition, as the Z-agars have to be made up in-house they would perhaps not be a very attractive choice for use in clinical or industrial settings.

In summary, chromID Pa outperformed the other agars in terms of specificity but incubation time, temperature and interpretation of colony colour needs to be further considered in order to increase sensitivity. Chromogenic media are expensive, but if chromID Pa affords presumptive identification of *P. aeruginosa*, the costs of further biochemical tests may be off-set, as previously suggested (Laine *et al.* 2009). PCN and PIA are currently widely used general selective media and have high sensitivity, but may permit the growth of other species commonly encountered in CF and from industrial settings. Even though increasing the incubation temperature to 42°C may improve specificity and PPV values, further biochemical tests and/or genotypic methods are required for accurate species identification when using these media. Z-agar had lower specificity than previously reported (Szita *et al.* 1998; Szita *et al.* 2007) and is likely to be unsuitable for application in clinical or industrial environments without further modification.

3.3.2 Acetamide media as enrichment media for *P. aeruginosa*

Enrichment media are valuable when trying to isolate an organism from a complex sample which may be present in low numbers, or sub-lethally injured. The enrichment step aims to increase the number of cells of the target organism, whilst simultaneously discouraging the growth of undesirable species. Acetamide-based media have been used for the enrichment of *P. aeruginosa* but different studies report different formulations with individual formulations not being evaluated extensively. In addition, growth in these studies was assessed only by looking at broth turbidity as an end result. To further investigate acetamide broths as enrichment media, growth dynamics were scrutinised over 72 hours. The effect of previously reported acetamide concentrations (2 to 20 g/L) were assessed, in addition to lower (1 g/L) and higher (up to 50 g/L) concentrations. Three groups of strains were investigated: 1) prototrophic *P. aeruginosa* strains (PAO1, RW109 and RW110), 2) auxotrophic strains (LESB58) and *P. aeruginosa* strains displaying weak growth on solid acetamide media (RW113), and 3) non-*P. aeruginosa* strains which grew weakly on solid acetamide media (*P. putida* RW112, *B. lata* 383 and *B. dolosa* LMG 18943). The base formulation for the acetamide broth (Z-broth) was taken from (Szita *et al.* 1998) as the most recent publication investigating acetamide media, and its solid counterpart (Z-agar) was used in the comparative agar study.

Overall increasing the acetamide concentration from 1 to 20 g/L did not inhibit the growth of *P. aeruginosa* strains and did not change the overall growth patterns of non-*P. aeruginosa* strains, with the exception of *B. lata* 383 whose maximum culture density reached dropped from 0.37 at 1 g/L to 0.07 at 20 g/L acetamide. This suggests that the response to increasing the acetamide concentration is species (and potentially even strain) specific, as *B. dolosa* LMG 18943 behaved very differently to *B. lata* 383. Both of these species, in addition to *P. putida* RW112, grew weakly on acetamide agar and were positive for the *ami* operon PCR (methods section 2.9.1.2). It is not known, however, if the operon in these strains is capable of producing and transporting a fully functional amidase enzyme to metabolise acetamide. Increasing the acetamide concentration from 20 to 50 g/L did have an inhibitory effect on all three strains tested, decreasing the maximum OD reached to the lowest levels observed during the experiment. When looking at maximum OD reached by all strains over 72 hours, 20 g/L acetamide is the least inhibitory toward *P. aeruginosa* strains and yet had the biggest knock down effect on *B. lata* 383, the only non-*P. aeruginosa* strain which reached an OD similar to the prototrophic *P. aeruginosa* strains at 4 g/L acetamide. The results of this study indicate that acetamide selectivity for *P. aeruginosa* is improved at 20 g/L compared to the 4 g/L used by (Szita *et al.*

1998), although investigation into the growth dynamics of a larger strain panel is needed to confirm this.

Auxotrophic *P. aeruginosa* strains have difficulty growing when acetamide is the sole carbon and nitrogen source, and previous studies have reported low numbers of *P. aeruginosa* strains unable to grow in acetamide-based enrichment media (Oberhofer and Rowen 1974). *P. aeruginosa* strains growing weakly on acetamide media have also previously been identified and this trait attributed to the production of a defective amidase or problems with regulation of amidase production (Clarke and Tata 1973). In the present study, very low levels of growth were observed for the known auxotroph *P. aeruginosa* LESB58 (Winstanley *et al.* 2009), and the weakly growing industrial strain *P. aeruginosa* RW113, in all acetamide concentrations. Increasing the concentration of acetamide from 4 to 20 g/L, however, marginally improved the growth of *P. aeruginosa* RW113, as measured by an increase in maximum OD from 0.12 to 0.14. This increase would probably not be sufficient to distinguish it from a non-*P. aeruginosa*, but increased cell numbers could improve the chance of detection if a further selection step is performed.

One of the main problems encountered with the solid acetamide media was that it was difficult to distinguish between weakly growing *P. aeruginosa* strains and non-*P. aeruginosa* strains. It was thought that the growth dynamics in broth may set these groups apart. Growth in Z-broth containing 20 g/L acetamide revealed that whilst certain non-*P. aeruginosa* strains (*P. putida* RW112 and *B. dolosa* LMG 18943) exhibited similar growth patterns to the auxotrophic *P. aeruginosa* strain LESB58, others, such as *B. lata* 383, could be similar to weakly growing *P. aeruginosa* strains, such as *P. aeruginosa* RW113. It would therefore be difficult to use growth curves to differentiate between *P. aeruginosa* and non-*P. aeruginosa* strains, and it is highly likely that auxotrophic *P. aeruginosa* strains would be missed altogether. Prototrophic *P. aeruginosa* strains could potentially be distinguished from non-*P. aeruginosa* strains by growth dynamics at 20 g/L acetamide but a larger strain panel would be needed to assess this comprehensively. Whether in solid or broth form, acetamide-based media may not be suitable for straightforward identification, but the growth curve work has indicated that with modification they could still be of use for enrichment.

3.4 Conclusions

The main conclusions from the work in this chapter were as follows:

1) An industrial collection comprising 105 isolates was successfully assembled and isolates were identified to the species level where possible. This collection was invaluable as a well characterised resource for the PhD and industrial microbiology.

2) The comparative agar study revealed that although chromID *P. aeruginosa* was the agar with the highest specificity, it lacked sensitivity. Overall PCN was found to be the most robust selective agar, re-enforcing that the current industry practice of using a cetrimide-based medium was suitable for the identification of *P. aeruginosa*.

3) Although acetamide-based media demonstrated low specificity in the comparative agar study, they showed promise as enrichment media. It is likely that auxotrophic *P. aeruginosa* strains will not be isolated with acetamide media but using a higher concentration of acetamide than (Szita *et al.* 1998) could enhance the growth of weakly growing *P. aeruginosa* strains and reduce the growth of non-*P. aeruginosa* strains.

The hypotheses proposed at the beginning of the chapter were rejected (hypothesis 1) and accepted (hypothesis 2):

Hypothesis 1: All five selective agars in the comparative study will perform equally well in terms of specificity and sensitivity.

Hypothesis 2: Acetamide media will permit the growth of *P. aeruginosa* strains and inhibit the growth of non-*P. aeruginosa* strains. **The findings are not as straightforward as this, however, with concentrations of acetamide above 20 g/L having an inhibitory effect on *P. aeruginosa* strains, and the growth of non-*P. aeruginosa* strains being inhibited to different extents. Further work with the acetamide media is required.**

4 Culture-independent analysis of contaminated industrial products: detection of *P. aeruginosa* and bacterial diversity

4.1 Introduction

Traditionally, detection, identification and characterisation of bacteria have been via culture-dependent methods which assess phenotype. These methods can include the observation of cellular morphology, growth characteristics on selective and differential media, (Jimenez *et al.* 1999b) antibiotic susceptibility and the subjection of the micro-organism to various biochemical assays (Johnsen *et al.* 1996). Often culture-based identification is time-consuming, labour intensive and expensive, requiring large volumes of media and reagents. Despite this, and probably owing partially to their ease of use, culture-dependent methods are still routinely used in diagnostics in medical and industrial settings (Orus and Leranoz 2005; van Belkum *et al.* 2013). An increasing number of culture-independent detection methods are being developed and are starting to be implemented in industry, including ATP-bioluminescence, impedance, flow cytometry and PCR-based assays (Jimenez 2001b) although this has mainly been limited to food microbiology (Maukonen *et al.* 2003; Postollec *et al.* 2011; van Belkum *et al.* 2013).

4.1.1 Culture-independent detection of *P. aeruginosa*

As an opportunistic pathogen and 'objectionable' organism within industrial products, the detection of *P. aeruginosa* is very important. Numerous techniques have been published for the culture-independent detection of *P. aeruginosa* including end-point PCR and qPCR; both SYBR Green-based qPCR (Jaffe *et al.* 2001; Qin *et al.* 2003; Motoshima *et al.* 2007; Choi *et al.* 2013) and hydrolysis probe based qPCR (Anuj *et al.* 2009) formats have been evaluated. To date, ten different genes/regions including 16S rRNA, 16S-23S spacer region rRNA, *oprL*, *fliC*, *ecfX*, *gyrB*, *toxA*, *algD* and the O-antigen acetylase gene (Table 1.4) have been targeted. The *oprL* gene encodes an outer membrane lipoprotein and primers designed for the amplification of this gene by (De Vos *et al.* 1997) have been the most widely evaluated; the *oprL*-PCR has been applied to a range of samples, including pure culture (De Vos *et al.* 1997; Anuj *et al.* 2009), clinical (De Vos *et al.* 1997; Pirnay *et al.* 2000; Xu *et al.* 2004; Deschaght *et al.* 2011), environmental (Schwartz *et al.* 2006; Lavenir *et al.* 2007), and industrial samples (Jimenez *et al.* 1999b; Jimenez *et al.* 2000b; Jimenez 2001c; Farajnia *et al.* 2009). With the exception of the 16S-23S spacer region rRNA gene and the O-acetylase gene PCR (Choi *et al.* 2013) which have each only been evaluated by one study, none of the afore mentioned *P. aeruginosa*-specific PCRs have both 100% sensitivity and specificity. The continued increase in the number of available genome sequences and tools

for comparative genomic analyses provide the opportunity to enhance target gene identification and guide PCR primer design for the culture-independent detection of bacteria (Choi *et al.* 2013). This chapter used genetic information to develop two novel species-specific PCRs for *P. aeruginosa*, and assess their application to the detection of *P. aeruginosa* in HPC products

4.1.2 Culture-independent techniques to detect contamination of HPC products

Detection of bacterial contamination in HPC-products is routinely culture-based, but culture-independent PCR-based methods for the specific detection of low levels of microorganisms, including 'objectionable' bacterial (*Salmonella* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Staphylococcus aureus*) and fungal (*Aspergillus niger*) species have also been described (Jimenez *et al.* 1998; Jimenez *et al.* 1999a; Jimenez *et al.* 1999b; Jimenez *et al.* 2000a; Jimenez *et al.* 2000b; Jimenez *et al.* 2000c; Merker *et al.* 2000; Jimenez 2001c; Jimenez *et al.* 2001; ŠKof *et al.* 2004; Karanam *et al.* 2008; Farajnia *et al.* 2009). All of these studies included a culture-dependent, broth enrichment step prior to DNA extraction with the purpose of increasing bacterial cell numbers to detectable levels (Jimenez 2011). Whilst this step may be useful for the detection of non-fastidious species present as low level contaminants, enrichment may introduce a culture bias (Dunbar *et al.* 1997) and neglects the detection of viable but non-cultivable (VBNC) (Oliver 2005) cells. Additionally, these studies all used nutrient rich enrichment media which could miss fastidious or sub-lethally injured cells (Orus and Leranoz 2005) having more specialised nutritional requirements for revival. Direct nucleic acid extraction from products would circumvent these issues but presents a number of challenges.

HPC and pharmaceutical product formulations are complex, including raw materials such as surfactants, oils, waxes, cream bases, emulsifiers, humectants, plasticisers and preservatives, and vary depending on product application (Williams and Schmitt 1996). It is generally unknown whether certain product components can interfere with DNA extraction or inhibit PCR, as there is only evidence of two studies which have attempted this (Samadi *et al.* 2007; Farajnia *et al.* 2009). In these cases, DNA was extracted via treatment of the samples with a lysis buffer, followed by either a boiling step (Samadi *et al.* 2007) or phenol-chloroform extraction and ethanol precipitation (Farajnia *et al.* 2009). Universal bacterial and species-specific end-point PCRs were successfully applied to the DNA extracted from a pharmaceutical topical lotion (Samadi *et al.* 2007) and diluted expectorant syrup (Farajnia *et al.* 2009) with detection levels of approximately 10^2 cfu/ml and <10 cfu/ml, respectively. These studies suggest that an enrichment step may not be necessary if DNA extraction procedures are optimised. Much further work needs to be done, however, to determine whether different HPC products are also amenable to culture-independent analysis.

4.1.3 Bacterial diversity in industry

Currently we have an idea of the range of bacterial and fungal species encountered as contaminants in HPC-products through published reports of publicly available data (Wong *et al.* 2000; Jimenez 2007; Lundov and Zachariae 2008; Sutton and Jimenez 2012). These reports illustrate that although numerous species are found as contaminants, individual incidents are usually reported as caused by one or two species. Not all incidents detail which species are responsible, instead reporting generally as 'microbial or bacterial contamination'. To date, our knowledge of microbial diversity in HPC products has been solely based on culture-dependent techniques. It is widely regarded that total microbial diversity cannot be comprehensively assessed with culture, as the majority of microorganisms cannot be isolated on media (Ward *et al.* 1990; Armougom and Raoult 2009). Smaller scale culture-based diversity studies have been attempted in an industrial setting, such as that of La Duc *et al.* (La Duc *et al.* 2007) who assessed microbial contamination in a clean-room. In this study growth conditions were tailored for the isolation of physiologically diverse microbial populations, and molecular identification of the isolates was performed to the species level. Even robustly designed, broad culture studies are subject to growth biases and limitations and would be enhanced by simultaneous culture-independent analyses (Maukonen *et al.* 2003).

Culture-independent techniques that are commonly used in microbial community analysis include PCR-denaturing gradient gel electrophoresis (DGGE), length heterogeneity PCR (LH-PCR), ribosomal intergenic spacer analysis (RISA), sequencing of 16S rRNA gene fragments, and metagenomics (Maukonen *et al.* 2003; Rastogi and Sani 2011). As one of the main concerns in industry is the detection of specific problematic species, few studies have applied these community sampling techniques to investigate industrial microbial contamination (Maukonen *et al.* 2003). There have been diversity surveys of manufacturing environments, including water systems used in pharmaceutical manufacturing processes (Kawai *et al.* 1999; Kulakov *et al.* 2002; Kawai *et al.* 2004) and in the food and beverage industry, where food products (Rudi *et al.* 2004; Juste *et al.* 2008a) and factories (Timke *et al.* 2005) have been investigated (Juste *et al.* 2008b). In these applications, culture-independent techniques have provided a more in-depth analysis of the microbial complement of the environments (Rudi *et al.* 2004), identified differences in bacterial populations following treatment/production and storage (Kawai *et al.* 2004), uncovered genera likely to be in high abundance but which are considered uncultivable (Timke *et al.* 2005), and given an idea of the functionality of the contaminants by identifying genes associated with enzymatic activities (Kulakov *et al.* 2002). These findings could potentially inform industrial practices such as the design of manufacturing processes, the monitoring of process hygiene, decontamination operations, and provide information about

potential sources of microbial contamination (Maukonen *et al.* 2003; Juste *et al.* 2008b). As of yet, there have been no studies dedicated to the investigation of the bacterial diversity in HPC-products. Within this chapter, both culture-dependent and independent techniques were used to examine a panel of contaminated HPC-products belonging to five different product categories to further knowledge in this area.

4.1.4 Aims

The aims of this chapter were as follows:

1) To design and evaluate novel species-specific PCRs for the culture-independent detection of *P. aeruginosa*, and apply these to nucleic acids extracted from HPC-products

2) To assess the bacterial diversity associated with contaminated HPC-products, comparing both culture-dependent and –independent techniques

Hypothesis 1: Direct DNA extraction and detection of *P. aeruginosa* can be achieved from HPC products

Hypothesis 2: The bacterial diversity associated with HPC products is low

4.2 Results

4.2.1 Design and evaluation of species-specific PCRs for *P. aeruginosa*

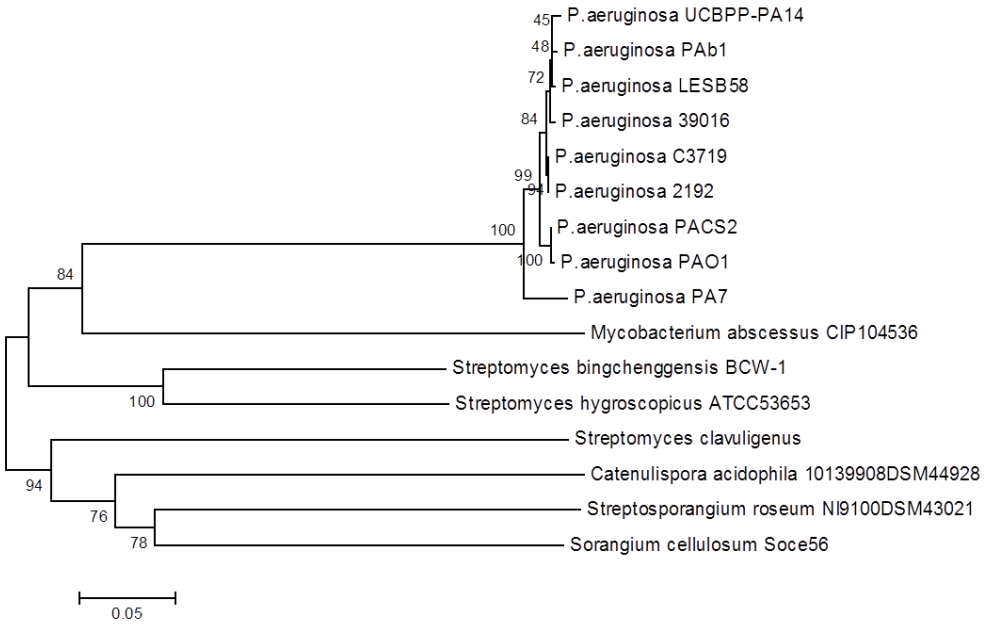
4.2.1.1 *phzS* gene PCR

The gene *phzS* encodes a flavin containing mono-oxygenase enzyme involved in the biosynthesis of pyocyanin, a pigment produced only by *P. aeruginosa* (Haynes 1951). Phylogenetic trees constructed from alignments of *phzS* genes 9 *P. aeruginosa* strains and *phzS* gene orthologs from 7 species within the Class *Actinobacteria* revealed the *phzS* gene to be well conserved among *P. aeruginosa* strains (Figure 4.1). Based on *phzS* and ortholog gene alignment, two sets of primers were designed (Figure 4.2). The first set of primers (*phzS*.F, 5'-GGAAGCCGGCAACGCCTA T-3' and *phzS*.R, 5'-GCAGGATCAACTGGTTGCGGGT-3') amplified a 584 bp fragment of the *phzS* gene, whilst the second set of primers (*phzS*N.F, 5'-CGCGGCGAACTGCAGATGATC-3' and *phz*SN.R, 5'-CTCGTTGTCGAGCTGGCCGA-3') were nested within the boundaries of the first set and amplified a 435 bp fragment.

4.2.1.2 *ami* operon PCR

In *P. aeruginosa* genes within the *ami* operon are involved in the biosynthesis and transport of an acylamidase enzyme. Both intergenic regions and genes within the operon were considered when designing *P. aeruginosa*-specific PCR primers. The *amiL-amiE* intergenic region was chosen as a target sequence for forward primer design as it was present in all 9 *P. aeruginosa* strains on the IMG database and orthologous regions were only present in one *Pseudomonas* species (2 strains of *P. putida*; Figure 4.3). The *amiE* gene was chosen for reverse primer design, being well conserved in *P. aeruginosa* strains (Figure 4.1) and having orthologs with sufficient sequence variability in other *Pseudomonas* species (Figure 4.3). The primers, *ami*E.F (5'-CATCAGGTCRTGCGCATCAGC-3') and *amiE*.R (5'-GCCCTTCGGCCCKTCGCT-3'), amplified a 588 bp fragment spanning the *amiL-amiE* intergenic spacer region and the *amiE* gene.

(A) *phzS* gene



(B) *amiE* gene

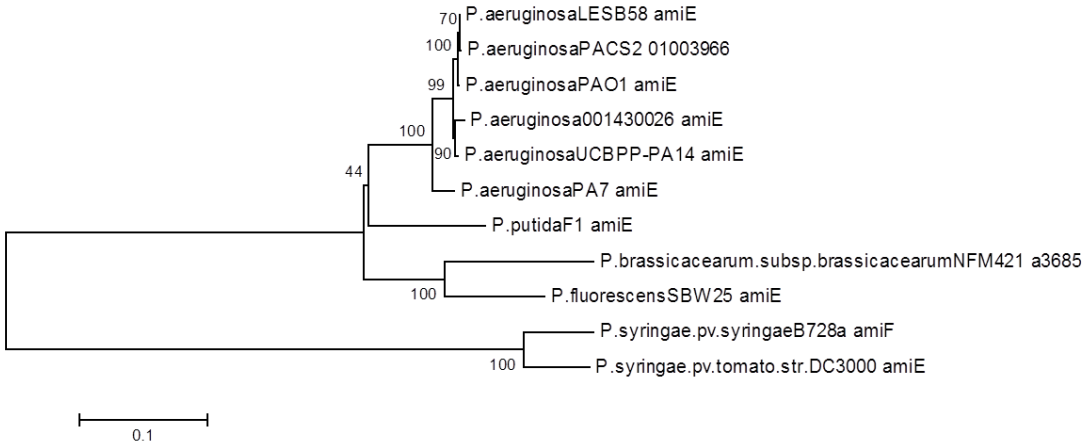


Figure 4.1 Sequence diversity of *P. aeruginosa phzS* and *amiE* genes and orthologous genes of other bacterial species.(A) *phzS* and *phzS* ortholog gene sequences, (B) *amiE* and *amiE* ortholog sequences. Gene sequences were aligned using ClustalW2 and a neighbour-joining phylogenetic tree based on 1000 bootstrap replicates constructed using the Jukes-Cantor model in MEGA6. Bootstrap values are shown at the nodes as percentages of 1000 replications. The trees are drawn to scale with genetic distances given as the number of base substitutions per site.

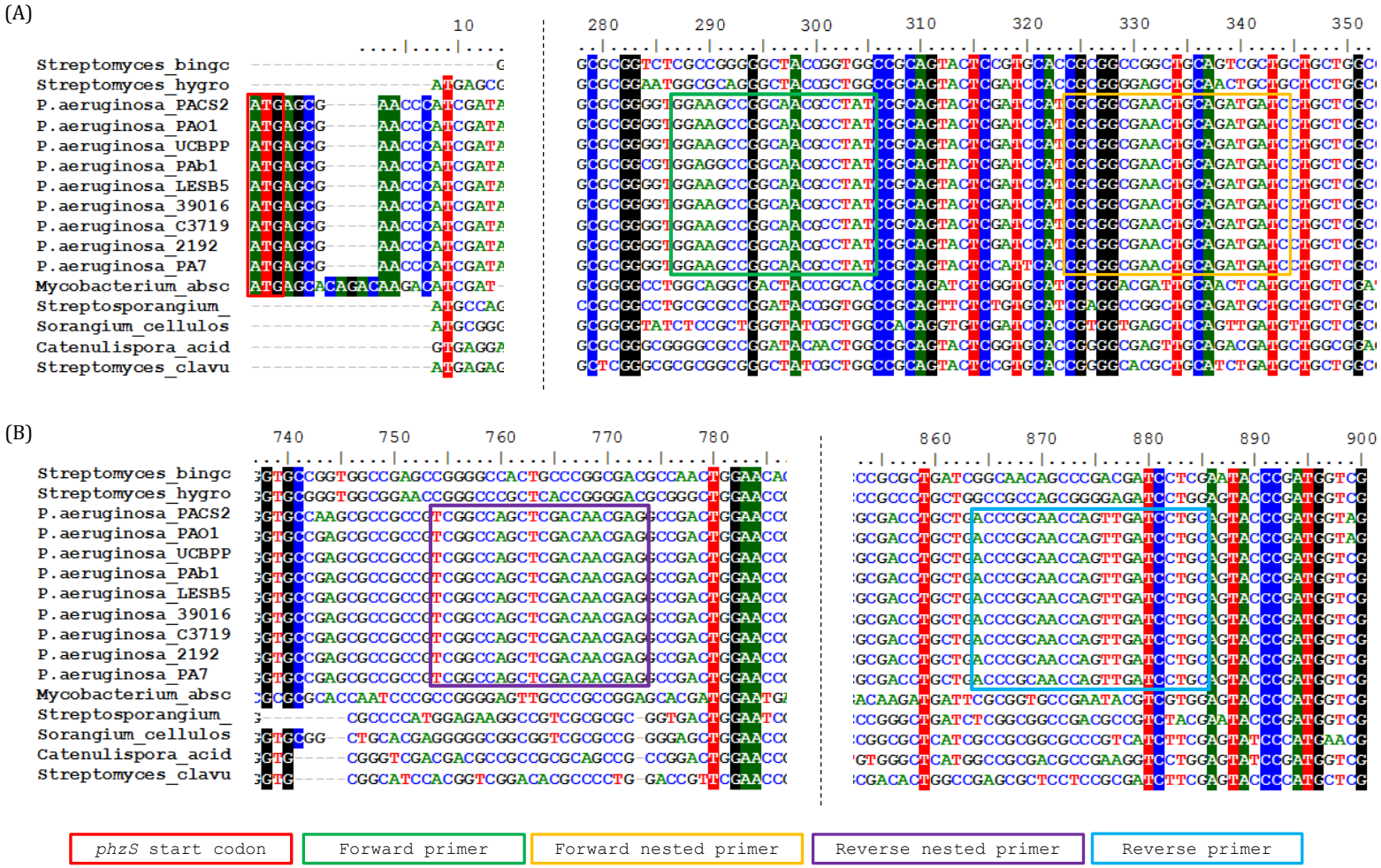

(A)
10
280
290
300
310
320
330
340
350
Streptomyces_bingc
Streptomyces_hygro
P.aeruginosa_PACS2
P.aeruginosa_PAO1
P.aeruginosa_UCBPP
P.aeruginosa_PAb1
P.aeruginosa_LESB5
P.aeruginosa_39016
P.aeruginosa_C3719
P.aeruginosa_2192
P.aeruginosa_PA7
Mycobacterium_absc
Streptosporangium_
Sorangium_cellulos
Catenulispora_acid
Streptomyces_clavu
(B)
740
750
760
770
780
860
870
880
890
900
Streptomyces_bingc
Streptomyces_hygro
P.aeruginosa_PACS2
P.aeruginosa_PAO1
P.aeruginosa_UCBPP
P.aeruginosa_PAb1
P.aeruginosa_LESB5
P.aeruginosa_39016
P.aeruginosa_C3719
P.aeruginosa_2192
P.aeruginosa_PA7
Mycobacterium_absc
Streptosporangium_
Sorangium_cellulos
Catenulispora_acid
Streptomyces_clavu
phzS start codon
Forward primer
Forward nested primer
Reverse nested primer
Reverse primer

Figure 4.2 Sequence alignments for *phzS* gene primer design. Sequences of the *phzS* gene from 9 *P. aeruginosa* strains and *phzS* gene orthologs from 7 non-*P. aeruginosa* species were aligned using ClustalW and viewed in BioEdit to facilitate primer design. The coloured boxes (see key) indicate the following: the *phzS* start codon, forward and nested forward primer locations (A), and the reverse and nested reverse primer locations within the *phzS* gene (B).

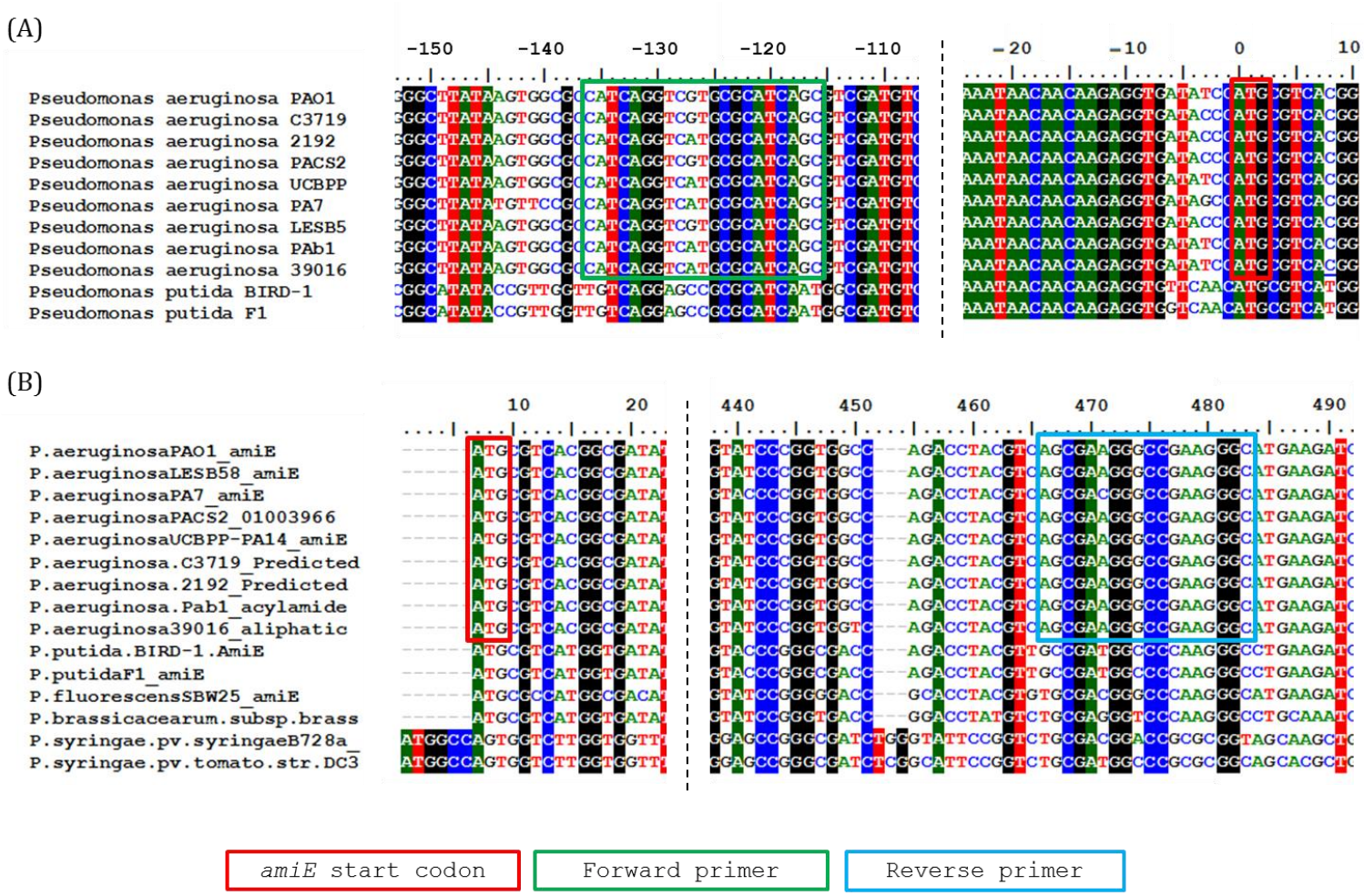


Figure 4.3 Sequence alignments for *ami* operon primer design.Sequences of the *amiL-amiE* intergenic region (A) and the *amiE* structural gene (B) from *P. aeruginosa* and orthologous regions from other *Pseudomonas* species were aligned using ClustalW and viewed in BioEdit to facilitate primer design. The location of the *amiE* start codon, forward and reverse primers are shown by the coloured boxes corresponding to the key.

4.2.1.3 Sensitivity and specificity of single round *phzS* and *ami* operon PCRs

The *phzS* and *ami* PCRs were evaluated using DNA extracted by the Chelex® method (section 2.6.1) from pure cultures of 60 *P. aeruginosa* and 65 non-*P. aeruginosa* strains (Table 2.6). Four further strains from a previous study (Finnan *et al.* 2004) that were found to be negative for *phzS* were also included in the panel for the new *phzS* PCR (Table 2.8). All *P. aeruginosa* strains were also confirmed as positive for the *oprL* PCR as to have confidence in species identity. Both PCRs demonstrated 100% sensitivity as positive results were observed for all of the *P. aeruginosa* strains screened. The specificity of the *phzS* and *ami* PCRs were 100% and 82%, respectively. The *ami* PCR gave an amplification product of the correct size (recorded as a false positive) for strains of *B. ambifaria* (n=1), *B. cepacia* complex (n=1), *B. contaminans* (n=1), *B. dolosa* (n=1), *B. lata* (n=1), *B. multivorans* (n=1), *B. pyrrocinia* (n=1), *P. mendocina* (n=1), *P. oleovorans* (n=1), *P. pseudoalcaligenes* (n=1), *P. putida* (n=1) and *Pseudomonas* spp. (n=1) (Table 4.1). For this reason, only the *phzS* PCR was investigated further.

Table 4.1 Specificity of species-specific PCRs for *P. aeruginosa*

Species and strains	Number of positive PCR reactions	
	***phzS* PCR**	***ami* PCR**
Achromobacter xylosoxidans		
LMG 1863	0	0
***Acinetobacter* species**		
UL-1L	0	0
***Bacillus* species**		
RW#162	0	0
Burkholderia ambifaria		
AMMD	0	1
Burkholderia cenocepacia		
RW#156	0	0
J2315	0	0
***Burkholderia cepacia* complex**		
UL-2P	0	1
UL-3P	0	0
Burkholderia contaminans		
LMG 23255	0	1
Burkholderia dolosa		
LMG 18943	0	1
Burkholderia gladioli		
BCC238	0	0
Burkholderia lata		
383	0	1
Burkholderia multivorans		
ATCC 17616	0	1*
Burkholderia phymatum		
BCC1607	0	0
Burkholderia phytofirmans		
BCC 1604	0	0
Burkholderia pyrrocinia		
LMG 21824	0	1
Burkholderia stabilis		
LMG 14294	0	0
Burkholderia thailandensis		
E264	0	0
Burkholderia vietnamiensis		
RW#152	0	0
G4	0	0
Enterobacter gergoviae		
UL-5P	0	0
***Enterobacter* species**		
UL-4L	0	0
Escherichia coli		
NCTC 12241	0	0
***Halomonas* species**		
RW#171	0	0
***Klebsiella* species**		
UL-13	0	0
Ochrobactrum anthropi		
RW#160	0	0
Pandoraea apista		
LMG 16407	0	0
Pandoraea pnomenusa		
LMG 18087	0	0
Pandoraea pulmonicola		
LMG 18106	0	0
Pandoraea sputorum		
LMG 18819	0	0
Pantoea agglomerans		

RW#166	0	0
Pseudomonas alcaligenes		
LMG 1224	0	0
Pseudomonas aureofaciens		
LMG 1245	0	0
Pseudomonas fluorescens		
RW#85	0	0
LMG 1794	0	0
Pseudomonas mendocina		
LMG 1223	0	1*
Pseudomonas oleovorans		
LMG 2229	0	1
Pseudomonas pseudoalcaligenes		
UL-9L; RW#112	0	1
Pseudomonas putida		
RW#153	0	0
RW#154	0	0
RW#157	0	0
RW#158	0	0
RW#159	0	0
RW#161	0	0
RW#205	0	0
LMG 2257	0	1*
Pseudomonas resinovorans		
LMG 2274	0	0
***Pseudomonas* species**		
RW#155	0	0
RW#163	0	0
RW#164	0	0
RW#165	0	0
RW#167	0	0
RW#169	0	0
RW#206	0	1
UL-10L; RW#111	0	0
Pseudomonas stutzeri		
LMG 11199	0	0
Ralstonia insidiosa		
LMG 18101	0	0
Ralstonia mannitolytica		
LMG 18103	0	0
Ralstonia picketii		
LMG 18088	0	0
Ralstonia respiraculi		
LMG 21510	0	0
Staphylococcus aureus		
ATCC 6538	0	0
NCTC 12981	0	0
Staphylococcus epidemidis		
ATCC 12228	0	0
Stenotrophomonas maltophilia		
LMG 958	0	0
***Stenotrophomonas* species**		
UL-12	0	0
Total number of positive PCR reactions/total number of strains	**0/65**	**12/65**
Specificity (%)	**100%**	**82%**

*A weak positive PCR result was observed

4.2.2 Culture-independent detection of *P. aeruginosa* in HPC-products

4.2.2.1 DNA extraction from HPC-products

An automated nucleic acid extraction robot, the Maxwell® 16 system, was investigated as a standardised kit-based system for DNA extraction from HPC products. A product sample input of either 0.3 ml (liquid product) or 0.3 g (viscous product) yielded between 0.077 and 8.41 ng/µl of DNA for 10 of the contaminated HPC products tested (Table 4.2). The DNA yield for the remaining 4 products, SC1, SC3, PC2 and WC1, was too low to quantify (less than 0.01 ng/µl). No correlation between DNA yield and the total viable counts obtained from the products (correlation coefficient = 0,153, p-value=0.574; Goodman and Kruskal's gamma rank correlation test for non-parametric data including 'tied' observations) (Goodman and Kruskal 1979).

To determine whether PCR inhibitors were carried over from HPC-products during the DNA extraction process a 16S rRNA gene PCR using universal bacterial primers was performed (section 2.7.1). As the concentrations of DNA obtained were low, undiluted DNA was used and the amount of template DNA in the PCR was increased to 6 µl (rather than 2 µl of 10 ng/µl DNA as used in a standard 16S rRNA PCR; section 2.7.1.1). Only three of the product samples (DWL4, SC1 and FWS3) did not yield a PCR amplification product (Table 4.2). Both DWL4 and FWS3 had sufficient concentrations of total DNA to yield a positive PCR result, 0.689 and 0.586 ng/µl respectively, suggesting the presence of PCR inhibitors, an absence of bacterial DNA or amplification below the level visible conventional agarose gel electrophoresis. The negative result for SC1, may have been due to insufficient DNA for the PCR reaction (<0.01 ng/µl). Overall, the Maxwell® 16 system was effective in extracting DNA from a range of different HPC product formulations which was of high enough quality and quantity for use in PCR applications.

4.2.2.2 Application of species-specific PCRs for *P. aeruginosa*

To identify the presence of *P. aeruginosa* in contaminated HPC-products, two *P. aeruginosa*-specific PCRs were applied to DNA extracted from products. Both the previously published *oprL* PCR (De Vos *et al.* 1997) and the *phzS* PCR detected *P. aeruginosa* in 9 out of 14 HPC-products, 8 of which were culture positive for *P. aeruginosa* (Table 4.2). The surface cleaner SC2 was culture-negative but yielded a positive result for the 16S rRNA gene, *oprL* and *phzS* PCRs, suggesting either preservative neutralisation issues or the presence of viable but non-cultivable or dead cells. Since the *phzS* PCR gave the same results as the well-established *oprL* PCR, it was was further validated as a novel PCR for the detection of *P. aeruginosa*.

To examine the detection limits of a single round *phzS* PCR and a nested *phzS* PCR, the PCRs were applied to DNA extracted from Neat, 10^{-1} and 10^{-2} dilutions of DWL1 and Neat and 10^{-1} dilutions of PC1. The viable counts and DNA concentrations (obtained from 0.3 g of product) are shown in Table 4.3, and the PCR results shown in Figure 4.4. The lower detection limit for a single round *phzS* PCR was approximately 10^{2} viable cells for DWL1 (10^{-1} product dilution), and approximately 10^{3} viable cells for PC1 (undiluted product). The application of the second round nested *phzS* PCR improved the detection limits for both products to approximately 10^{1} viable cells for DWL1 and between 10^{1} and 10^{2} for PC1, which corresponded to the 10^{-2} and 10^{-1} product dilutions respectively. By cultivation (see section 4.2.3) DWL1 was contaminated with only *P. aeruginosa*, but PC1 contaminated with more than one species, with *P. aeruginosa* the dominant contaminant. The viable counts recorded for PC1 were for total cells and therefore may overestimate the number of *P. aeruginosa* cells and the amount of *P. aeruginosa* DNA present. However, the efficacy of the nested *phzS* PCR for the detection of at least 1000 viable *P. aeruginosa* cells was validated.

Table 4.2 Culture-dependent and –independent analysis of contaminated HPC-products

Product	Culture-dependent analysis			Culture-independent analysis				
	CFU/g[b]	Nº colony types	16S rRNA Species ID from colony types	DNA concentration[c] (ng/µl)	16S rDNA PCR	*oprL*/ *phzS* PCR[f]	RISA profile	Culture-independent 16S rDNA[g] Genus ID
Dish Wash Liquid								
DWL1[a]	10^3	1	*P. aeruginosa*	0.993	+	+/+	1 dominant band (Pa[d])	*Pseudomonas*
DWL2[a]	10^2	1	*P. aeruginosa*	0.103	+	+/+	Multiple bands (Pa[c])	*Pseudomonas* *Enterobacter* *Herbaspirillum*
DWL3[a]	10^3	2	*P. aeruginosa* *Enterobacter* spp.	0.623	+	+/+	Multiple bands	*Enterobacter* *Pseudomonas*
DWL4[a]	10^3	2	*Pseudomonas* spp.	0.689	-	-/-	No bands	*Pseudomonas* *Herbaspirillum*
DWL5[a]	10^4	1	*P. aeruginosa*	0.077	+	+/+	1 dominant band (Pa[d])	*Pseudomonas*
Surface Cleaner								
SC1	10^3	1	*P. aeruginosa*	<0.01	-	-/-		
SC2[a]	-	-	-	0.245	+	+/+	1 dominant band (Pa[d])	*Pseudomonas* *Herbaspirillum*
SC3	10^3	1	*Acinetobacter* spp.	<0.01	+	-/-		
Personal Care								
PC1[a]	10^3	2	*P. aeruginosa* *Enterobacter cloacae*	0.06	+	+/+	Multiple bands	*Enterobacter* *Pseudomonas* *Herbaspirillum*
PC2[a]	10^4	1	*P. aeruginosa*	<0.01	+	+/+	1 dominant band (Pa[d])	*Pseudomonas* *Herbaspirillum*
Fabric Wash								
FWS1[a]	10^4	2	*P. aeruginosa*[e]	1.53	+	+/+	1 dominant band (Pa[d])	*Pseudomonas*
FWS2[a]	10^6	1	*P. aeruginosa*	8.41	+	+/+	1 dominant band (Pa[d])	*Pseudomonas*
FWS3	10^4	1	*Pseudomonas* spp.	0.586	-	-/-		
Wood Cleaner								
WC1	10^4	1	*Burkholderia* spp.	<0.01	+	-/-		

[a]Included in the RISA and culture-dependent 16S rRNA analysis; [b]Viable counts performed on TSA medium; [c]DNA concentration in 300 µl or 0.3 g of product; [d]A band of approximately 753 bp was taken as indicative of the presence of *P. aeruginosa*; [e]*P. aeruginosa* colony types were the same RAPD type and putatively considered same-strain; [f]A positive PCR result (+) was taken as a visible band on a gel as indicated in Figure 4.1; [g]16S rRNA gene amplification and pyrosequencing carried out by Research and Testing Laboratory

Table 4.3 Viable counts and DNA yield for the HPC-products DWL1 and PC1

Product dilution	CFU/0.3 g	DNA concentration in 0.3 g (ng/μl)
DWL1		
Neat	10^3	0.993
10^{-1}	10^2	0.209
10^{-2}	10^1	<0.01 (Too low to detect)
PC1		
Neat	10^3	0.06
10^{-1}	10^1 - 10^2	<0.01 (Too low to detect)

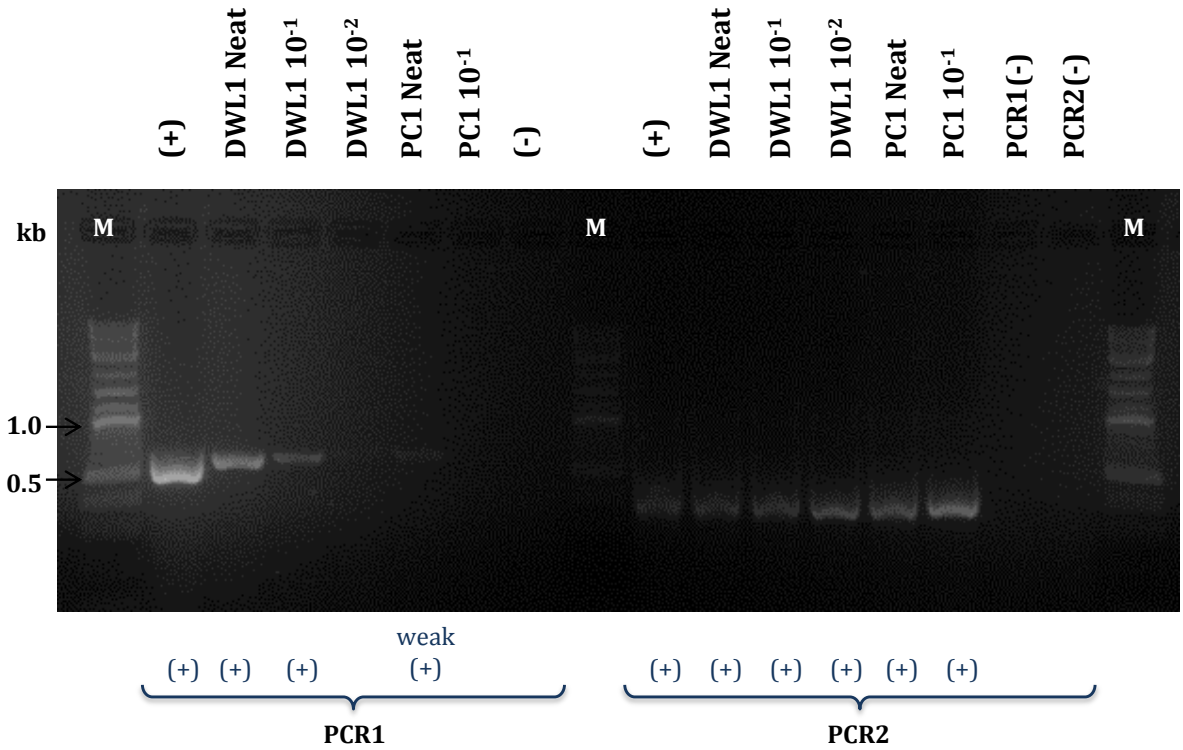


Figure 4.4 Detection limits of the *phzS* PCR. Lanes: M, molecular ladder (relevant marker size indicated in kb); (+), *P. aeruginosa* PAO1 positive control; DWL1/PC1 dilutions, DNA extracted from Neat, 10^{-1} or 10^{-2} product dilutions; (-), negative water controls. The PCR1 bracket indicates the results from a single round *phzS* PCR and the PCR2 bracket indicates the results from a nested *phzS* PCR, which takes the DNA template as the products from PCR1.

4.2.3 Bacterial diversity associated with HPC-products

4.2.3.1 Identification of cultivable bacteria

After growth on TSA, 15 different colony morphologies were isolated from 13 HPC-products, with SC2 failing to yield any bacterial growth (Table 4.2). Identification of each colony type was performed using 16S rRNA gene sequencing and analysis (Table 4.2). For 9 of the products, only 1 colony type was isolated. In these cases the contaminating organisms were *P. aeruginosa* (DWL1, DWL2, DWL5, PC2, and FWS2), *Pseudomonas* spp. (FWS3), *Acinetobacter* spp. (SC3) and *Burkholderia* spp. (WC1). For 4 of the remaining products, 2 different colony morphotypes were identified from each. In the case of DWL3 and PC1, the colony types were determined to be *P. aeruginosa* and an *Enterobacter* spp.. The two different colony morphologies were isolated from DWL4 were both *Pseudomonas* spp., but could not be identified below the genus level. FWS1 was contaminated with two *P. aeruginosa* isolates displaying different colony morphologies. RAPD PCR genotyping showed that these colony types shared the same RAPD fingerprint and were therefore putatively considered as the same strain. Although the surface cleaner, SC2, failed to yield any bacterial growth, cultivation-independent analysis with the *oprL* and *phzS* PCRs identified that *P. aeruginosa* DNA was present in the sample (Table 4.2).

4.2.3.2 Bacterial diversity profiling by RISA

The genetic fingerprinting technique RISA was applied directly to DNA extracted from 10 of the 14 HPC product samples. Of the 10 products, 6 had a single dominant band (DWL1, DWL5, SC2, PC2, FWS1 and FWS2), three had multiple bands (DWL2, DWL3 and PC1), and 1 had no profile (DWL4) (Figure 4.2). To aid the putative identification of contaminants, DNA from a pure culture of *P. aeruginosa* PAO1 was subjected to RISA PCR. The 753 bp product obtained from the *P. aeruginosa* RISA PCR correlated with the *in Silico* PCR tool predictions of the size of ITS regions from this species. The dominant band observed for DWL1, DWL5, SC2, PC2, FWS1, FWS2, and DWL2 also correlated to the ITS amplicon size for *P. aeruginosa*. The *P. aeruginosa* ITS band was missing from the PC1 and DWL3 profiles, in which the dominant band correlated to ITS sizes predictive of *Enterobacter* (625-640 bp). A weaker band of approximately 625-640 bp was also observed for DWL2. As the *In Silico* PCR tool predicted multiple ITS amplicons of different sizes for *Enterobacter* species, however, and multiple bands in a similar size range for other closely related species (e.g. *Pantoea* spp.), RISA profiles could not be definitively linked to the presence of *Enterobacter* within PCR1, DWL2 and DWL3. Overall though, it was clear that there was greater species diversity in the products PC1, DWL3 and DWL2 than the other

products, reflected by their more complex RISA profiles composed of at least 3 amplified ITS fragments (Figure 4.2).

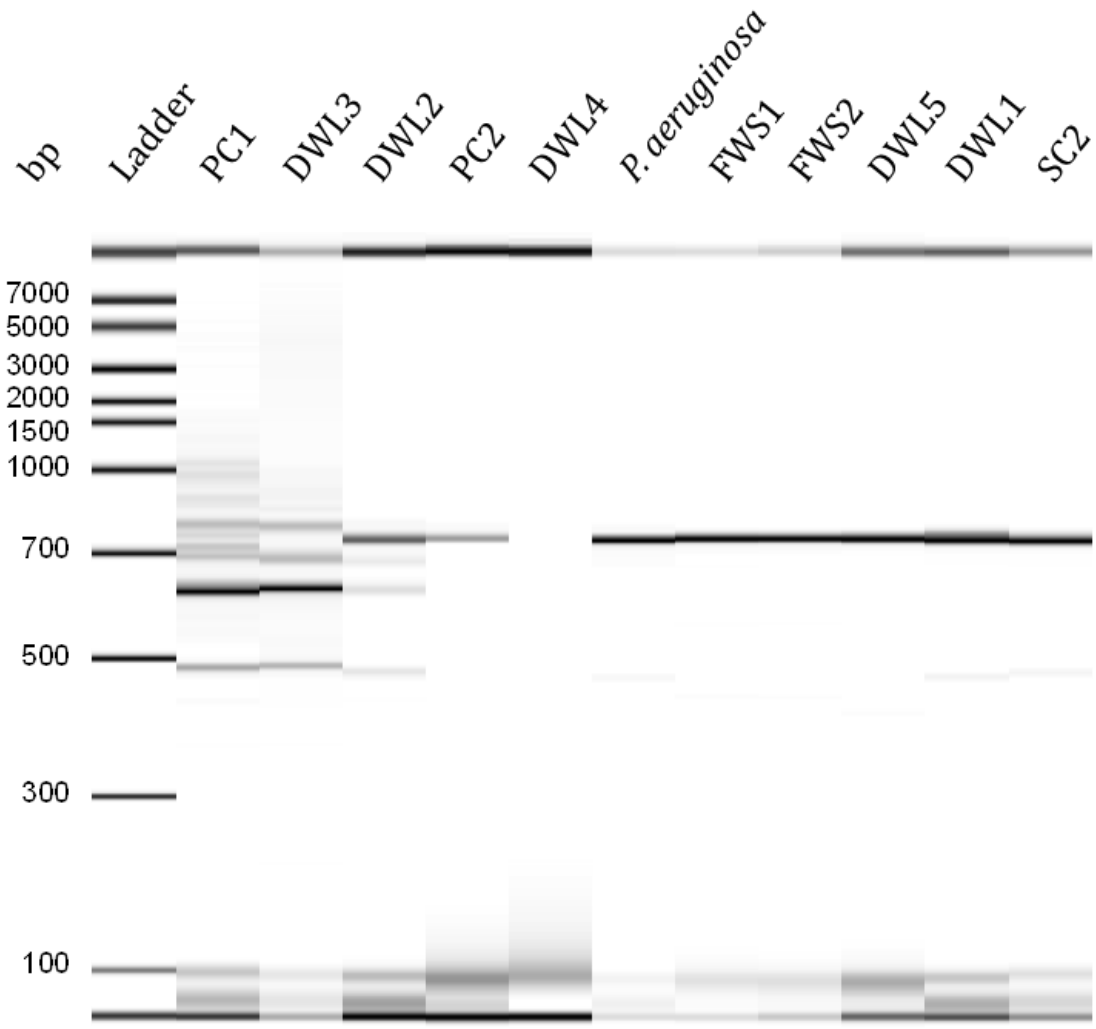


Figure 4.5 RISA profiles from contaminated HPC-products. The RISA profiles of 10 HPC-products are shown alongside the ITS region amplicon (753 bp) for a pure culture of *P. aeruginosa* strain PAO1 and a molecular ladder to aid band sizing (size indicated in bp).

4.2.3.3 Bacterial diversity by 16S rRNA gene pyrosequencing and analysis

16S rRNA gene amplification, pyrosequencing and analysis using the software package MOTHUR was used to comprehensively assess the bacterial diversity in 10 contaminated HPC-products. Amplification of the 16S rRNA gene for pyrosequencing was successful for all products including DWL4, suggesting that the process carried out by Research and Testing Laboratory was more sensitive. The number of sequence reads per sample ranged from 1763 (PC1) to 9301 (FWS2). Sub-sampling to the lowest number of sequence reads (1763) was performed. Throughout the analyses operational taxonomic units (OTUs) were grouped based on 97% sequence similarity.

From the primary analysis of sequences using MOTHUR and the 454 SOP pipeline (Table 4.4) the OTU richness was found to be low, with number of observed species (Sobs) at the 97% sequence similarity level ranging from a minimum of 6 (PC2) to a maximum of 15 (DWL1, SC2 and PC1). Both the Shannon and Inverse Simpson (InvSimpson) indices take into account the richness and evenness of OTUs to give an indication of how the diversity within a sample is spread. Across the 10 samples the Shannon diversity ranged from 0.055 (DWL5) to 0.723 (DWL3), and the InvSimpson diversity values ranged from 1.01 (FWS1, FWS2 and DWL5) and 1.650 (DWL3). These values are very low, with the Shannon diversity values being close to 0 and the InvSimpson diversity values close to 1, suggesting that most of the abundance was concentrated within one or a few OTUs for each sample. The Good's coverage estimates, which indicate the probability that a randomly selected sequenced from a sample has already been sequenced, were above 99% for all of the HPC product samples indicating that coverage was high and sufficient to detect the genera present.

Taxonomic assignments to the genus level were made using a combination of the RDP Classifier script and manual analysis using the RDP II seqmatch function. As stipulated in Methods (section 2.12.2), an OTU threshold was implemented and OTUs excluded if they were represented less than 10 times across all of the 10 HPC-product samples. Initially, 86 OTUs representing 30 genera were identified which were filtered to a final total of 6 OTUs belonging to the genera *Pseudomonas*, *Enterobacter* and *Herbaspirillum*. All of the products had either *Pseudomonas* (DWL1, DWL2, DWL4, DWL5, SC2, PC2, FWS1 and FWS2) or *Enterobacter* (DWL3 and PC1) as the dominant contaminating genus, where the proportion of total reads for that genus was over 80%. *Herbaspirillum* sequence reads never comprised more than 0.5% of total sequence reads for a product (Table 4.5 and Figure 4.3).

Table 4.4 Sequence and OTU diversity of the 16S rRNA gene 454 pyrosequencing datasets from the 10 contaminated HPC-products.

Product	Total reads	Subsampled reads	OTU richness*	Diversity indices		Good's Coverage (%)
			Observed species numbers (Sobs)	Shannon	InvSimpson	
Dish Wash Liquid						
DWL1	5567	1763	15	0.112	1.017	99.3
DWL2	2841	1763	13	0.195	1.058	99.5
DWL3	4456	1763	7	0.723	1.650	99.8
DWL4	2131	1763	14	0.239	1.082	99.5
DWL5	2576	1763	7	0.055	1.010	99.7
Surface Cleaner						
SC2	7397	1763	15	0.164	1.04	99.5
Personal Care						
PC1	1763	1763	15	0.516	1.265	99.5
PC2	3224	1763	6	0.075	1.020	99.8
Fabric Wash						
FWS1	4725	1763	11	0.078	1.01	99.4
FWS2	9301	1763	9	0.063	1.01	99.5

*Outputs from analysis using the software package MOTHUR and the 454 SOP pipeline at 97% similarity

Table 4.5 Genus distributions of 16S rRNA sequence reads recovered from ten contaminated HPC-product samples

Product	Number of sequence reads (% total)		
	Pseudomonas	***Enterobacter***	***Herbaspirillum***
Dish Wash Liquid			
DWL1	1750 (100)	-	-
DWL2	1720 (98.1)	29 (1.7)	3 (0.2)
DWL3	316 (18)	1443 (82)	-
DWL4	1746 (99.8)	-	4 (0.2)
DWL5	1758 (100)	-	-
Surface Cleaner			
SC2	1746 (99.94)	-	1 (0.06)
Personal Care			
PC1	177 (10.1)	1562 (89.4)	8 (0.5)
PC2	1759 (100)	-	-
Fabric Wash			
FWS1	1754 (100)	-	-
FWS2	1756 (100)	-	-

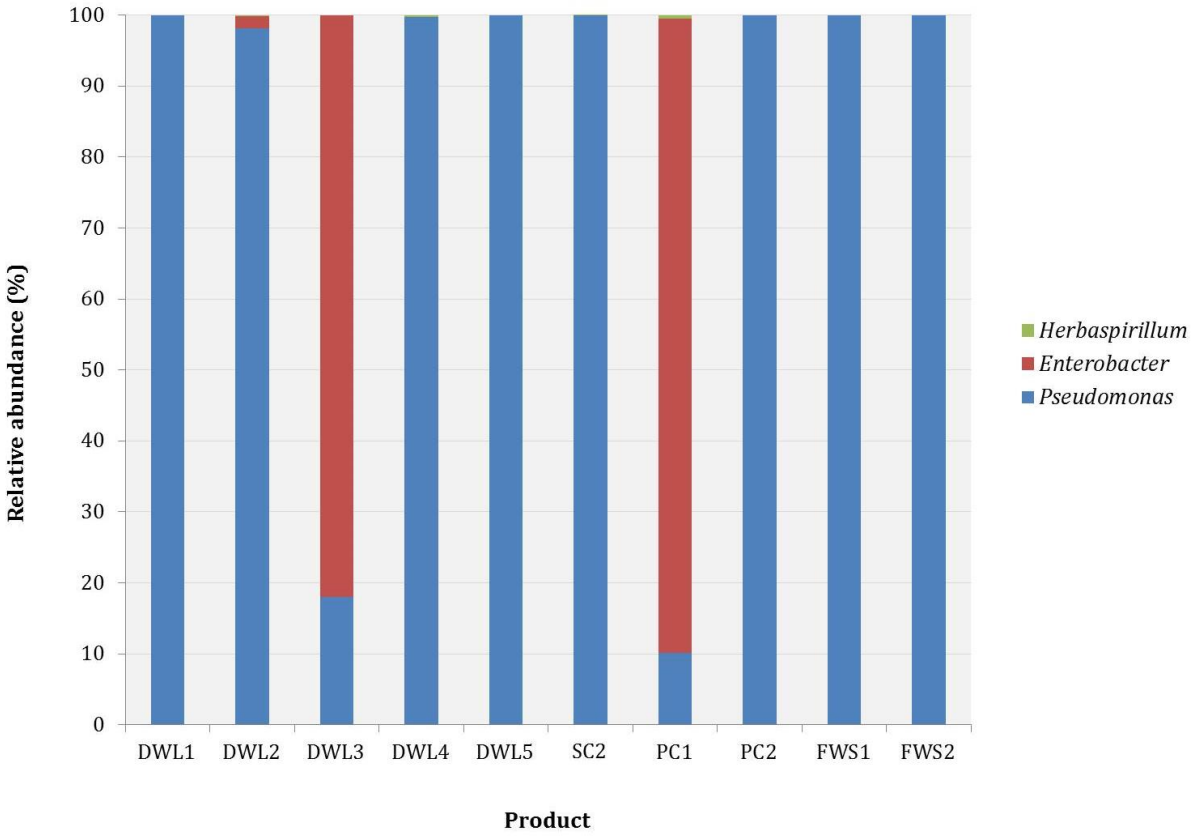


Figure 4.6 Genus distributions of 16S rRNA sequence reads recovered from ten contaminated HPC-product samples depicted graphically.

4.2.3.4 Comparison of culture-dependent and -independent methods for identifying species diversity

The results from the culture-dependent and culture-independent assessments of diversity in contaminated products are shown side-by-side in Table 4.2. Comparisons could be made for the products DWL1, DWL2, DWL3, DWL4, DWL5, SC3, PC1, PC2, FWS1 and FWS2, as these had results for both culture-dependent and -independent analyses. All of the contaminated products which were culture positive for *P. aeruginosa* were also positive for the *P. aeruginosa*-specific *oprL* and *phzS* PCRs. The RISA profiles for all but two of these products, DWL3 and PC1, had a dominant band of approximately 753 bp corresponding to the predicted *P. aeruginosa* ITS amplicon. The RISA PCR may have not produced an amplicon for DWL3 and PC1 as the total number of 16S rRNA pyrosequencing reads corresponding to *P. aeruginosa* was low in these products, 18% and 10.1%, respectively. The culture-independent 16S rRNA genus IDs were in concordance with the culture-dependent work, corroborating contamination with *Pseudomonas*.

DWL2, DWL3, DWL4 and PC1 were contaminated with species other than *P. aeruginosa*. DWL4 was found to be contaminated with non-*P. aeruginosa Pseudomonas* spp. by both culture dependent methods and 16S rRNA gene pyrosequencing and analysis. DWL2, DWL3 and PC1 had multiple bands in their RISA profiles and were found to be contaminated by *Enterobacter* spp., in addition to *P. aeruginosa*, by culture-independent methods. In the case of DWL2, *Enterobacter* was missed by culture dependent techniques; 16S rRNA gene pyrosequencing and analysis indicated that the proportion of sequence reads for *Enterobacter* in DWL2 was only 1.7%. *Herbaspirillum* was found in DWL1, DWL2, DWL4 and PC1 by 16S rRNA gene analysis (less than 0.5% of total pyrosequencing reads) but not identified by RISA or in culture dependent investigations. With these last two exceptions, and the absence of the *P. aeruginosa* ITS amplicon in the DWL3 and PC1 RISA-PCR profiles, the culture-dependent and –independent investigations yielded concordant results for bacterial contaminant identification.

4.3 Discussion

4.3.1 Novel species-specific PCRs for the culture-independent detection of *P. aeruginosa*

Culture-independent methods for the detection of *P. aeruginosa* have been widely detailed and 10 different genes commonly targeted for PCR-based identification (Table 1.4). Amongst these, the *oprL* gene PCR has been extensively evaluated and applied to the detection of *P. aeruginosa* from varied environments. Despite this widespread use, the *oprL* PCR does not have 100% specificity, and increasing numbers of *P. aeruginosa* genome sequences determined there was an opportunity for improved PCR primer design. Two novel PCRs were designed which targeted the *phzS* gene and genes within the *ami* operon. Primer design was informed by the alignment of multiple gene sequences from *P. aeruginosa* strains and closely related species. The *phzS* gene is involved in the biosynthesis of pyocyanin, a pigment produced exclusively by *P. aeruginosa.* As the *phzS* gene is highly conserved amongst *P. aeruginosa* strains and few orthologs exist in other species it was considered a suitable PCR gene target. There are no published species-specific PCRs for the detection of *P. aeruginosa*, although PCRs have been designed for the amplification of *phzS* as a 'virulence' gene (Finnan *et al.* 2004; Bradbury *et al.* 2010). Both studies used the primers designed by (Finnan *et al.* 2004) which amplify a 1752 bp fragment of the *phzS* gene and determined that a number of *P. aeruginosa* isolates were PCR negative for this gene; 5/17 (Finnan *et al.* 2004) and 4/184 (Bradbury *et al.* 2010). If a proportion of *P. aeruginosa* isolates do not have the *phzS* gene, this would have implications for the sensitivity of any PCRs targeting this region. The newly designed *phzS* PCR described in this chapter had different primer sequence targets designed from systematic alignment of available *P. aeruginosa* gene sequences and amplified a smaller region of the gene (584 bp), than those designed to detect *phzS* as a virulence gene (Finnan *et al.* 2004).

Screening against a panel of 60 *P. aeruginosa* isolates and 65 non-*P. aeruginosa* isolates determined the *phzS* PCR to have 100% sensitivity and specificity. This panel was systematically selected to include genetically distinct *P. aeruginosa* strains and strains of *Pseudomonas* and non-*Pseudomonas* species often misidentified as *P. aeruginosa* or commonly encountered in cystic fibrosis microbiology (Spilker *et al.* 2004; Hauser *et al.* 2011). The *phzS* PCR showed a significant advantage over the *ami* PCR in not producing false postives with certain *Burkholderia* and other *Pseudomonas* species. The 5 *P. aeruginosa* isolates previously found by (Finnan *et al.* 2004) to be negative for *phzS* were also tested. Of the 5 isolates, one was *oprL* and *phzS* PCR negative (strain BR220), therefore potentially a non-*P. aeruginosa* species, whilst the remaining 4 (strains CF194, CF198, CF242 and BR227) were positive for the newly

designed *phzS* PCR. Thus far, the *phzS* gene appears to be a reliable target for a species-specific PCR to identify *P. aeruginosa* and the newly designed primers have excellent sensitivity and specificity in a single round PCR.

Genes within the *ami* operon were also targeted for novel PCR design, and primers were designed to amplify a 588 bp fragment of the operon spanning the *amiL-amiE* intergenic region and the structural *amiE* gene. This operon encodes the AmiE aliphatic amidase enzyme which is well conserved in *P. aeruginosa* strains and is responsible for the hydrolysis of acetamide (Brammar and Clarke 1964). The *ami* PCR displayed 100% sensitivity, but a much lower specificity (82%) with strains of 5 *Pseudomonas* and 7 *Burkholderia* species giving a positive PCR result. Other species, such as *Pseudomonas* and *Burkholderia*, are known to possess amidase enzymes which can metabolise acetamide (Szita *et al.* 1998). The results of Chapter 3 also illustrated that acetamide-based media have low selectivity and may permit the growth of species other than *P. aeruginosa*. The *ami* primers in this study were designed based on alignments of the *P. aeruginosa* intergenic spacer region, the *amiE* gene and orthologous regions/genes from other species, but unfortunately lacked specificity. Other PCRs designed specifically for the amplification of the *amiE* gene of *P. aeruginosa* have also been reported to amplify an amidase gene from *B. cepacia* (Novo *et al.* 2003). This suggests that there may not be enough variability between the amidase genes of different species to design a highly specific PCR.

4.3.2 Application of culture-independent techniques to industrial microbiology

Direct bacterial DNA extraction from cosmetics and HPC-products has only been described by two studies (Samadi *et al.* 2007; Farajnia *et al.* 2009), where two different product types and two different DNA extraction methodologies were used. The majority of PCR-based analysis of products has previously been achieved through a broth enrichment step from which DNA was extracted (Jimenez 2011). Whilst this was successful, it is not a completely culture-independent approach. In this chapter a method for extracting DNA directly from a variety of HPC-product formulations was described. The DNA yields were relatively low (<0.01 to 8.41 ng/µl), but sufficient for a positive in-house 16S rRNA gene PCR for 11 out of the 14 products (Table 4.2). The remaining three products (DWL4, SC1 and FWS3) belonged to different HPC product categories indicating a potentially sample specific issue, rather than a problem with a certain product technology. A lack of correlation between the total viable counts and the amount of DNA extracted was also observed, and could have been influenced by either culture dependent (insufficient product neutralisation) or culture-independent (DNA extraction inhibitors, PCR inhibitors, detection of dead cells) limitations. To accurately assess DNA recovery yields from

products, experiments would need to be conducted with sterile products spiked with known numbers of viable cells, as has been described previously (Farajnia *et al.* 2009). Although further work is required to optimise DNA recovery from certain HPC product types, the Maxwell 16® system successfully handled a variety of product types, offering a straightforward, fast, standardised protocol for DNA extraction.

The application of species-specific PCRs to DNA extracted from products facilitates rapid detection of problematic species or opportunistic pathogens, which HPC products must be devoid of. The option of a multiplex PCR, such as that described by (Farajnia *et al.* 2009) would allow the simultaneous detection of more than one species, further improving on reporting times. In this chapter, both the *oprL* PCR and the newly designed *phzS* PCR were used to detect *P. aeruginosa* in contaminated HPC samples. In almost all of the products where *P. aeruginosa* was found using culture-dependent techniques (DWL1, DWL2, DWL3, DWL5, PC1, PC2, FWS1 and FWS2), the *oprL* and *phzS* PCRs also gave a positive result. The exceptions were SC2, where no results were obtained for culture but a PCR positive was obtained, and SC1 where *P. aeruginosa* was found by culture but culture-independent analysis was unsuccessful.

The *phzS* PCR and a nested *phzS* PCR was also applied to DNA extracted from serial dilutions of two products, DWL1 and PC1. Correlation to the viable counts from the serial dilutions indicated that a single round PCR could detect 10^2 viable cells in DWL1 and 10^3 cells in PC1, improving to 10^1 and 10^1-10^2, respectively, after the nested PCR. It is not known, however, if the DNA extracted from the products is representative of viable cells, or a mixture of viable and dead cells, which could lead to a lower-than-actual detection limit being recorded. Conversely, PC1 was contaminated with *P. aeruginosa* (as the dominant species), in addition to *Enterobacter* which may have given a slightly higher-than-actual detection limit, as the total DNA and colony counts would have been representative of the two species. Further work with samples artificially spiked with known numbers of *P. aeruginosa* cells could give more accurate detection limits for the *phzS* PCRs.

Whilst having numerous advantages over culture-dependent methods, culture-independent methods are not without their drawbacks. The detection of dead cells or free DNA in the product may over-estimate the level of contamination, and components within the product may inhibit either DNA extraction or PCR. (Maukonen *et al.* 2003; Juste *et al.* 2008b). Although the latter problem may be dealt with via the optimisation of protocols, other culture-independent techniques offer solutions to assessing viability. The inclusion of certain chemicals into PCR reactions, including ethidium monoazide which binds to DNA within dead cells, can facilitate the amplification of DNA from only viable cells (Rudi *et al.* 2005). It is also possible to target RNA,

rather than DNA, as an indicator of viability, as demonstrated by (Brown *et al.* 2009). Here, reverse transcriptase PCR was used to detect the presence of Mycoplasma in biopharmaceutical products. As RNA is more sensitive to degradation than DNA, the feasibility of extracting RNA from HPC-samples would need to be assessed; the Maxwell® 16 system has kits for total RNA extraction which could be tested in future. Overall, it is likely that a combination of both culture-dependent and –independent approaches would be the most practical and beneficial to industry for the acquisition of reliable and comprehensive results (Juste *et al.* 2008b).

4.3.3 Bacterial diversity in HPC-products

The microbial diversity found in HPC products has not yet been systematically investigated. Reports of publicly available product recall data are the only source of information in this area (Jimenez 2007; Lundov and Zachariae 2008; Sutton and Jimenez 2012). To date, our knowledge of contaminants derives from isolation of species by culture-dependent techniques, and may therefore overlook VBNC and other uncultivable cells. In this chapter a combination of culture-dependent and -independent methods was employed to assess the bacterial diversity associated with a panel of HPC products belonging to five different product categories.

These investigations have corroborated previous culture-based findings that bacterial diversity is low in HPC products, with no more than a few species being found together in the same product. For the 10 products where both culture-dependent and –independent approaches could be applied, the consensus result was contamination with one dominant bacterial genus, where this genus represented at least 80% of the pyrosequencing reads. For the products DWL1, DWL2, DWL4, DWL5, SC2, FWS1 and FWS2, *Pseudomonas* was dominant, and for DWL3 and PC1, *Enterobacter* was dominant. DWL2 also contained *Enterobacter*, and products DWL3 and PC1 had *Pseudomonas* as lower level contaminants (less than 20% total pyrosequencing reads). In the case of DWL1, DWL2, DWL3, DWL4, SC1, PC1, PC2, FWS1 and FWS2 the *Pseudomonas* species was likely to be *P. aeruginosa*, whilst for DWL5 it was likely to be a non-*P. aeruginosa Pseudomonas* species. Only in the case of PC1 was the *Enterobacter* species putatively identified as *E. cloacae.* Four products (DWL2, DWL4, SC1 and PC1) also contained the genus *Herbaspirillum*, but this was only identified by pyrosequencing and was present at less than 0.5% of the total sequence reads for each product. Whereas *P. aeruginosa*, *Pseudomonas* spp.*,* and *Enterobacter* are commonly encountered contaminants of non-sterile products, there is no evidence in the FDA or EU RAPEX reports of *Herbaspirillum* being detected. It is possible that *Herbaspirillum* has been previously overlooked as a non-cultivable species present in products, or that it a contaminant in another capacity in the DNA extraction and

pyrosequencing processes. *Herbaspirillum*, in addition to numerous other species, can contaminate molecular biology grade water, PCR reagents and DNA extraction kits (Salter *et al.* 2014), indicating that the inclusion of a negative control samples would be useful and caution should be exercised when analysing sequence data from diversity studies. Ascertaining whether the low level of *Herbaspirillum* 16S rRNA gene reads (<0.05%) was the result of genuine HPC contamination or reagent contamination will be difficult to prove; since water is a major raw material in HPC manufacturing processes the presence of *Herbaspirillum* DNA is plausible. From the four remaining products *P. aeruginosa* (SC1), *Acinetobacter* spp. (SC3), *Pseudomonas* spp. (FWS3) and *Burkholderia* spp. (WC1) were isolated, all of which are previously reported contaminants, but further culture-independent analysis would be needed to confirm their identities.

Overall the results of both the culture-dependent and –independent investigations were very similar, with three notable exceptions: 1) DWL2 was found to contain *Enterobacter* by culture-independent techniques but not by culture; 2) *Herbaspirillum* was detected by pyrosequencing in DWL1, DWL2, DWL4 and PC1 but not found by culture or RISA, and; 3) DWL3 and PC1 were found to be contaminated with *P. aeruginosa* by culture, but no *P. aeruginosa* ITS band was observed in their RISA profiles. Where genera were missed by culture, the contaminants were likely to be at low levels, as reflected by low numbers of pyrosequencing reads, and may have been VBNC or sub-lethally injured, or simply process contaminants as discussed above. Potentially, the inclusion of more than one medium in the study could have increased the chance of isolation; for example, the low nutrient medium R2A has been reported to enhance the growth of slow growing or stressed bacteria (Reasoner and Geldreich 1985), yielding higher viable cell counts than other nutritionally rich media (Means *et al.* 1981; van der Linde *et al.* 1999). The discrepancies encountered with the RISA profiles could be indicative of low DNA concentrations for the less-dominant species and potentially linked to a PCR-amplification bias (Polz and Cavanaugh 1998). Whilst RISA can provide an overall idea of diversity through the generation of a community profile, it cannot unequivocally identify individual species and may over- or under-estimate richness; unrelated species can possess similar size ITS regions and individual species can generate multiple ITS amplicons of different sizes (Kovacs *et al.* 2010). In this chapter the products contaminated with 3 genera (DWL3 and PC1) did have the most complex RISA profiles correctly indicating more diversity than the other samples. Pyrosequencing gave valuable insight into the ratios of genera present in the samples, although could only identify contaminants to the genus level due to the lack of resolution of the 16S rRNA gene (Armougom and Raoult 2009). Other methods utilised in this chapter, such as the species-specific PCRs and the isolation and identification of contaminants, provided additional

information which aided further taxonomic assignments. As both culture-independent and –independent techniques have advantages and limitations, a combination of both, in addition to careful interpretation, facilitates the most comprehensive and accurate results.

It should be mentioned that 8 out of the 14 products were initially selected for investigation as they were thought to be contaminated with *P. aeruginosa* (DWL1, DWL2, DWL3, DWL4, SC1, SC2, PC1 and PC2). Therefore, the products are not representative of the HPC industry as a whole, and neither are the overall ratios of contaminants. For example, in this study *P. aeruginosa*, *Pseudomonas* spp., *Enterobacter* and *Herbaspirillum* were found in 64%, 14%, 21% and 29% of the products, respectively, whilst in reality the respective incidences are 7-27%, 8%, 3-6%, 0% (Sutton and Jimenez 2012)(EU RAPEX reports 2005-2014). In addition *Burkholderia* spp., found in one out of the 14 products, is one of the most common contaminants of non-sterile products, with an incidence rate of between 5-34% (Sutton and Jimenez 2012)(EU RAPEX reports 2005-2014). A prospective survey of HPC contamination incidents is required to fully understand microbial diversity associated with this manufacturing industry.

4.4 Conclusions

The main conclusions from the work in this chapter were as follows:

1) Two novel species specific PCRs were designed for the detection of *P. aeruginosa*. The *phzS* PCR demonstrated 100% specificity and sensitivity, and has potential as a new molecular diagnostic tool for *P. aeruginosa.*

2) Direct DNA extraction from HPC products was achieved using the Maxwell 16® system, yielding DNA of high enough quality and quantity for use in PCR for 11 out of the 14 products. This technology represents a straightforward, fast, standardised method for DNA extraction from complex samples such as HPC products.

3) The bacterial diversity associated with HPC products is low, with few species co-contaminating products. There was general concordance between the findings of the culture-dependent and –independent investigations, but a combination of both was necessary to obtain the most comprehensive view of diversity and accurate species identification.

The hypotheses proposed at the beginning of the chapter were accepted:

Hypothesis 1: Direct DNA extraction and detection of *P. aeruginosa* can be achieved from HPC products.

Hypothesis 2: The bacterial diversity associated with HPC products is low.

5. Genetic diversity of *P. aeruginosa* in industry

5.1. Introduction

Bacterial populations vary in genetic diversity and population structures are complex, ranging from highly clonal to non-clonal. The evolutionary driving forces of the population structures differ, with clonal populations generally evolving through *de novo* mutation, whilst genetic change in non-clonal populations results largely from recombination (Spratt and Maiden 1999). Populations may also take an intermediate structure, as has been suggested for *P. aeruginosa*, which is predominantly non-clonal but with evidence of prevalent clonal types (Pirnay *et al.* 2009). Overall, the majority of studies have shown that *P. aeruginosa* isolates are diverse and the population is freely-recombining but globally distributed major clone types have been identified, including Clone C and PA14 (Lomholt *et al.* 2001; Wiehlmann *et al.* 2007b; Pirnay *et al.* 2009; Maatallah *et al.* 2011; Dettman *et al.* 2013; Tümmler *et al.* 2014). In addition, in general, it appears that many *P. aeruginosa* strains exhibit little habitat selection and are genotypically, chemo-taxonomically and functionally highly similar (Nicas and Iglewski 1986; Römling *et al.* 1994; Rahme *et al.* 1995; Foght *et al.* 1996; Alonso *et al.* 1999). There are exceptions, however, indicating potential niche specialists within the population (Tümmler *et al.* 2014). These exceptions include the association of certain serotypes with the nosocomial settings (Pirnay *et al.* 2009), genotypes with keratitis (Stewart *et al.* 2011), clones with cystic fibrosis patient groups (Lanotte *et al.* 2004; van Mansfeld *et al.* 2010), and clonal complexes within aquatic environments (Khan *et al.* 2008; Selezska *et al.* 2012) (Table 5.1).

The genome of *P. aeruginosa* strains varies between 5.2 and 7 Mbp and displays extensive genetic diversity (Schmidt *et al.* 1996). The genome has been described as a mosaic structure, composed of the core genome, which is conserved (0.5-0.7% sequence diversity) and estimated to comprise approximately 4000 genes, and the variable accessory genome (Klockgether *et al.* 2011). The accessory genome accounts for up to 20% of the total genome content and is associated with horizontal gene transfer, harbouring many genes involved in mobility, metabolism, virulence and antibiotic resistance (Rumbaugh 2014). It is the accessory genome which accounts for the majority of the diversity between *P. aeruginosa* strains, and is found in regions of genomic plasticity interspersed throughout the chromosome (Klockgether *et al.* 2011).

Genotyping schemes utilise genetic information to investigate strain diversity and population biology. A variety of typing schemes have been used for *P. aeruginosa* (see Table 1.5), which

have sampled different proportions and parts of the genome. Examples of these schemes include genome fingerprinting techniques such as PFGE (Morales *et al.* 2004) or RAPD-PCR (van Mansfeld *et al.* 2010), sequence based methods such as MLST (Maatallah *et al.* 2011; Kidd *et al.* 2012) and the micro-probe array based method Clondiag Array Tube (AT) typing (Wiehlmann *et al.* 2007b; Cramer N. *et al.* 2012). Other studies have assembled polyphasic profiles of genotypic and phenotypic characteristics, which were interrogated by biological data analysis software (Pirnay *et al.* 2002; Pirnay *et al.* 2005) or minimum spanning tree analysis (Pirnay *et al.* 2009). More recently, whole genome sequencing (WGS) has also been used as a means to examine *P. aeruginosa* population biology (Dettman *et al.* 2013). Whilst the different typing techniques have advantages and disadvantages, the general consensus from all of the studies has been that *P. aeruginosa* has a non-clonal epidemic population structure (Table 5.1).

The majority of studies using genotyping techniques to assess *P. aeruginosa* population biology have focussed on isolates from clinical settings, with only a few looking specifically at environmental isolates (Pirnay *et al.* 2005; Khan *et al.* 2008; Selezska *et al.* 2012). Studies investigating a variety of sources have also only included environmental isolates in small proportions, representing between 10-25% of the isolate collection (Selezska *et al.* 2012). Such a bias towards clinical isolates could potentially give an unrealistic view of the population structure (Spratt and Maiden 1999). Furthermore, the genotypes of *P. aeruginosa* isolates associated with industry have not yet been surveyed. In addition to gaining further insight into population biology, genotyping of isolates associated with contamination incidents may be useful to industrial practices (White *et al.* 2011). It is currently unknown whether certain strains are more likely to associate with different product types and it would be advantageous to uncover potential sources of contamination and identify strains causing recurrent contamination problems. Few studies have typed bacterial contaminants of HPC products, however a study by Kutty *et al.* successfully linked human infection with strains of *B. cenocepacia* to contaminated mouthwash (Kutty *et al.* 2007). As at present *P. aeruginosa* strains are largely considered to be ubiquitous, it would be interesting to determine whether industrial isolates have distinct genotypes, share the same genotypes as those linked with infection or are associated with multiple habitats.

Table 5.1 Studies investigating the population structure of *P. aeruginosa* and their major findings

Genotyping technique		Study collection	Population structure/comments	Reference
Clondiag Array Tube (AT)-typing		240 isolates of cystic fibrosis, clinical and environmental origin	Non-clonal epidemic population structure. The majority of strains belonged to a few dominant clone types widespread in disease and environmental habitats The most common genotype was PA14.	(Wiehlmann *et al.* 2007b)
		134 isolates from patients with chronic obstructive pulmonary disease (COPD) and the isolates from Wiehlmann *et al.* (Wiehlmann *et al.* 2007b)	No *P. aeruginosa* clone types were found to be associated with COPD. The most prevalent global clone types were also found as the most abundant within the COPD population	(Rakhimova *et al.* 2009)
		63 isolates from patients with keratitis and a database of strains from three previous studies (Wiehlmann *et al.* 2007a; Mainz *et al.* 2009; Rakhimova *et al.* 2009)	Association of a subgroup of *P. aeruginosa* isolates with keratitis	(Stewart *et al.* 2011)
		381 environmental strains from river systems in North Germany	Certain clonal complexes were found to be associated with different water qualities in the river systems	(Selezska *et al.* 2012)
		454 strains from cystic fibrosis patients from 51 centres in Europe and Australia, 501 strains of clinical and environmental origin (955 strains in total)	Non-clonal epidemic population structure. No link between clone types and environmental and little evidence for a widespread transmissible cystic fibrosis clone. The most common clone types were clone C and PA14.	(Cramer N. *et al.* 2012)
Multi-Locus Sequence Typing (MLST)		143 isolates of clinical and environmental origin	Non-clonal epidemic population structure	(Curran *et al.* 2004)
		34 isolates of clinical and environmental origin. Environmental isolates from the open ocean, coastal areas and freshwater environments in Japan.	Certain genotypes were found to be associated with the open ocean	(Khan *et al.* 2008)
		110 strains from 5 Mediterranean countries and from clinical and environmental sources	Non-clonal epidemic population structure	(Maatallah *et al.* 2011)
		501 isolates of cystic fibrosis, clinical, animal and environmental origin	Non-clonal epidemic population structure. Limited association between genotype and ecological setting	(Kidd *et al.* 2012)
Multi-Locus Variable-number tandem-repeat analysis (MLVA)		363 isolates of cystic fibrosis and clinical origin	Population structure was found to be highly diverse and population specific. Clones from non-CF patients were distinct from clones from CF patients. Transmission of clones between different groups is rare.	(van Mansfeld *et al.* 2010)
Random Amplified Polymorphic DNA (RAPD)-PCR typing		162 isolates of cystic fibrosis and clinical origin	Associations were found between RAPD-PCR groups and CF or non-CF isolates	(Lanotte *et al.* 2004)
Analysis of a composite data set	Nucleotide sequences of three outer membrane protein genes i) *oprI*, ii) *oprL*, iii) *oprD,* iv) Amplified-Fragment Length Polymorphism (AFLP), v) serotype, vi) pyoverdine type [Bionumerics biological data analysis software used]	73 clinical and environmental isolates collected mainly in the late 1980s and 1990s globally	Non-clonal epidemic population structure	(Pirnay *et al.* 2002)
	i) *oprL* gene sequence, ii) ALFP, iii) serotype, iv) pyoverdine type, v) antibiogram including 21 MICs of clinically relevant antibiotics [Bionumerics biological data analysis software used]	100 isolates from the River Woluwe, Brussels, from water samples taken over 1 year	Non-clonal epidemic population structure	(Pirnay *et al.* 2005)
	i) O serotype, ii) Fluorescent Amplified-Fragment Length Polymorphism (FALFP) pattern, nucleotide sequences of three outer membrane protein genes, iii) *oprI*, iv) *oprL*, v) *oprD*, vi) pyoverdine receptor gene profile (*fpvA* type and *fpvB* prevalence), and prevalence of vii) exoenzyme genes *exoS* and *exoU* and viii) group I pilin glycosyltransferase gene *tfpO* [minimum spanning tree analysis used]	328 isolates collected over 125 years from 69 localities in 30 countries, of human, animal and environmental origin	Non-clonal epidemic population structure. A core lineage was predominant in both disease and environmental habitats globally. Although no evidence was found for a widespread transmissible cystic fibrosis clone, the study found the multi-drug resistant serotype O12 clone was associated with the nosocomial environment	(Pirnay *et al.* 2009)
	The presence of the virulence factors *lasA*, *lasB*, *exoS*, *exoT*, *exoU* and *ctx*, and zymography of staphylolysin, elastase and alkaline protease	69 isolates from patients with different eye infections, 76 of clinical and environmental origin (145 in total)	Non-clonal epidemic population structure	(Lomholt *et al.* 2001)
Whole genome sequencing		32 isolates from cystic fibrosis patients	Non-clonal epidemic population structure. The population is composed of many unique recombining genotypes with the occurrence of successful epidemic clone types that can spread to a higher prevalence	(Dettman *et al.* 2013)

5.1.1. Aims

The aims of this chapter were as follows:

1) Investigate *P. aeruginosa* strain diversity associated with HPC products using RAPD-PCR typing

2) Examine the population biology of *P. aeruginosa* by the application of Clondiag AT genotyping and multi-locus sequencing typing (MLST)-based methods to a sub-panel of industrial strains

3) Determine whether *P. aeruginosa* genotypes display habitat selection

Hypothesis: *P. aeruginosa* has a non-clonal epidemic population structure and strains isolated from industrial settings share genotypes with strains of cystic fibrosis, clinical and environmental origin.

5.2. Results

5.2.1. RAPD-PCR genotyping of an industrial *P. aeruginosa* isolate collection

A collection of 69 *P. aeruginosa* isolates associated with industry was assembled (Table 2.10) and genotyped using RAPD typing (section 2.10.1.1). Within this collection were isolates from contaminated home and personal care products (n=65), along with reference strains used in industrial testing (n=4). Of the reference strains used in industrial testing, 1 was of clinical origin (RW151), 1 was of environmental origin (RW198) and 2 were of unknown origin (RW196 and RW197). A dendrogram illustrating the similarities between RAPD-PCR profiles the isolates is shown in Figure 5.1. Using a cut off of 80% profile similarity, above which isolates were considered same strain, 26 different strain types were identified by RAPD, indicated by the different branch colours on the dendrogram (Figure 5.1).

Three factors made it difficult to draw robust conclusions regarding any links between strain and isolation location or product type: 1) the collection comprised sequential isolates from the same location and same product type, as demonstrated by the large number of dish wash liquid isolates from Italy sharing the same RAPD type; 2) there was a bias toward isolates from dish wash liquid (DWL; n=41), with other product types being underrepresented, for example fabric wash (FWS; n=1) and liquid abrasive cleaner (LAC; n=3), and; 3) not all isolates had comprehensive background information (isolation date/isolation location/product type), with different amounts of information being available for each isolate.

Despite these limitations, the following traits were noted from the RAPD genotyping (Figure 5.1). Four different strain types were associated with products from Italy, RW130 (DWL isolate; Italy RAPD type 1), the cluster encompassing RW120, RW151, RW121, RW122, RW131, RW132, RW119, RW128, RW124, RW126, RW137, RW145, RW146, RW125 and RW174 RW129 (All DWL isolates except RW151; Italy RAPD type 2), RW138 (LAC isolate; Italy RAPD type 3) and RW129 (DWL isolate; Italy RAPD type 4). Within the 'Italy RAPD type 2' cluster, all of the isolates from Italy were isolated in 2010 except RW#146, which was isolated in 2004, suggesting a recurrent contaminating strain. One strain type was detected in DWL products from Indonesia, one from Thailand and three from Malaysia, and there were no overlaps between DWL strain types isolated from different geographic locations. In contrast, there was profile similarity between RW138 isolated from an LAC in Italy, and RW133 and RW144 which were both isolated from DWL products in Thailand. The same RAPD strain type was also observed to be associated with more than one product type, for the cluster containing RW138 (LAC product), RW144, RW133 and RW134 (DWL products), and RW115 (SC product).

However, the main trend from the RAPD analysis was to show that more than one strain type can be associated with the same product type. Interestingly, RW151 (a reference strain isolated from clinical settings) has the same profile as a strain type associated with DWL contamination in Italy (RW#131 and RW#146), demonstrating overlap between clinical and industrial RAPD types (Figure 5.1).

Using this dendrogram, 19 isolates were chosen for further analysis (see Figure 5.1, strains with black box outlines around the RW IDs). These isolates were chosen to be representative of the RAPD profile diversity of the industrial isolate collection. In general each selected isolate belonged to a different RAPD dendrogram cluster, with the exception of RW151 (reference strain, isolated from clinical settings), RW131 (DWL strain type, isolated from Italy in 2010) and RW146 (DWL strain type, isolated from Italy in 2004) in order to observe differences between isolates of the same stain which had been isolated from different sources and dates. Along with five other strains recovered from clinical and environmental sources (RW11, RW18, RW27, RW30 and RW99; see Table 5.2), the 19 isolates associated with industrial settings were subjected to AT genotyping and WGS, to gain further insight into the population biology of *P. aeruginosa* in industry.

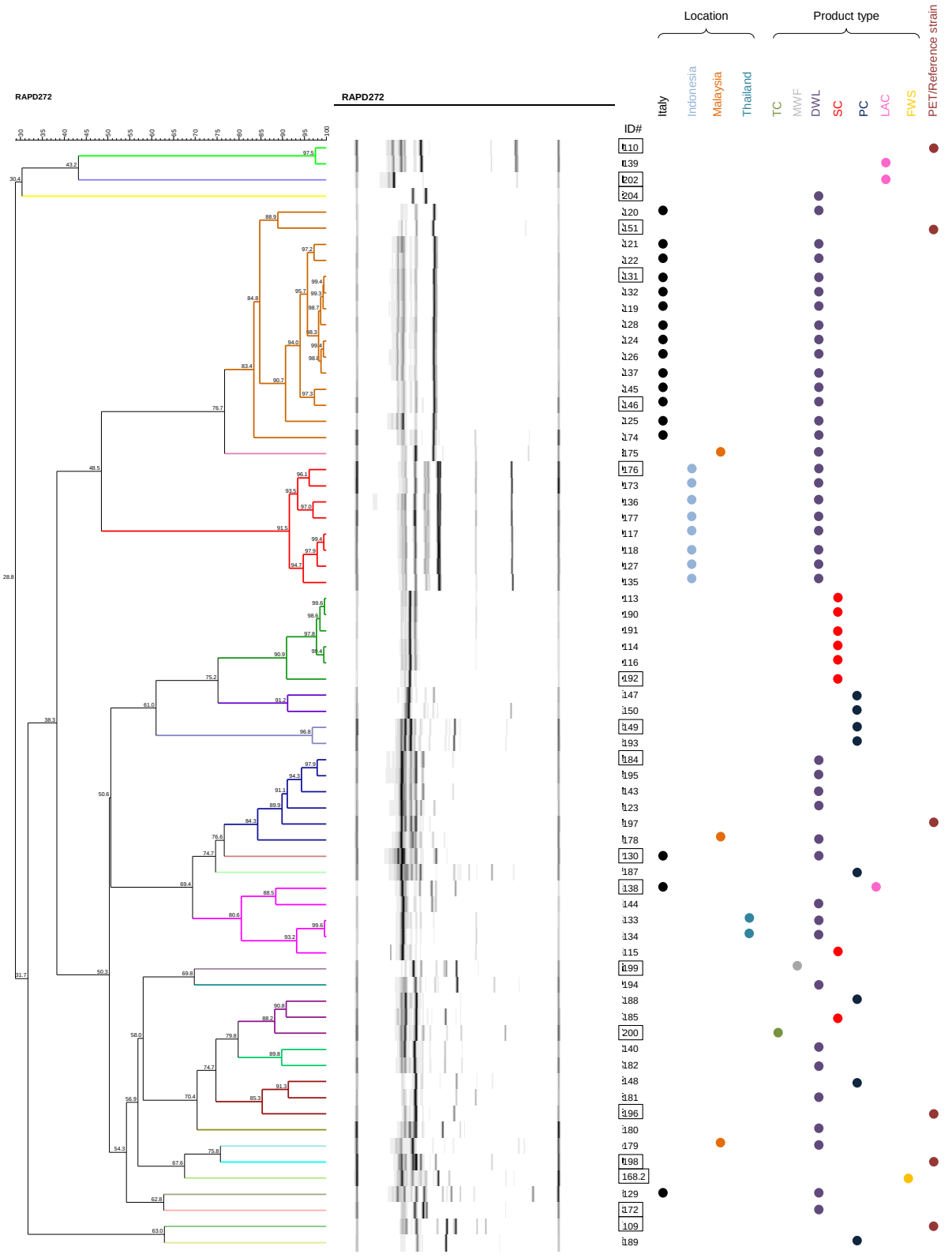


Figure 5.1 Clustered RAPD-PCR profiles of 69 industrial *P. aeruginosa* isolates. A Pearson correlation similarity coefficient was used to construct a UPGMA dendrogram of the RAPD-PCR profiles. The PCR fingerprint profile of each isolate is shown to the right of the dendrogram, along with the isolate number in the RW collection (ID#) and coloured coded information about isolate provenance. Percentage similarities are indicated by the scale bar and on the branches of the dendrogram. Profiles sharing ≥80% similarity were putatively considered same strain and different strain types are indicated by different coloured branches. Black boxes around ID numbers indicate isolates selected for Clondiag AT typing and genome sequencing. Preservative efficacy testing (PET) strains (IDs #109 and 110) and reference strains (IDs #151, 196, 197 and 198) are those used in antimicrobial efficacy testing in industry. Product type codes are follows: TC, timber care; MWF, metal working fluid; DWL, dish wash liquid; SC, surface cleaner; PC, personal care product; LAC, liquid abrasive cleaner; FWS, fabric wash.

5.2.2. Clondiag AT genotyping of a *P. aeruginosa* strain panel

A collection of 24 strains was genotyped using the AT genotyping system (Table 5.2; see section 2.10.2 for method). The strains originated from clinical (n=5), environmental (n=2), industrial (n=16) and unknown (n=1) sources. AT-genotypes were obtained for 23 out of the 24 strains, with no AT-genotype being recorded for the industrial strain RW168.2. The strains belonged to 18 different AT-genotypes as shown in Table 5.2. The 16 strains isolated from contaminated industrial products belonged to 11 different AT-genotypes. The AT-genotypes of the 23 strains were compared to a database containing 917 *P. aeruginosa* strains from diverse sources. This collection was a sub-set of the 955-strong panel used by Cramer *et al.* to investigate the diversity of cystic fibrosis *P. aeruginosa* isolates (Cramer N. *et al.* 2012); only strains from the panel which had complete 16-digit binary codes were used in the present analysis. Comparison with the database revealed that of the 18 different AT-genotypes, 5 were novel, with 4 of the novel genotypes belonging to industrial strains. The other AT-genotypes were shared with previously recognised clones or genotypes associated with different environments as elaborated in Table 6.

The eBURST algorithm grouped the 240 genotypes found from the collection of 940 strains into 8 small clonal complexes, one very large clonal complex and 45 singletons (Figure 5.2; only the 14 genotypes of industrial testing reference strains and strains from contaminated products are highlighted in the eBURST diagram). 740 (79% of the collection) strains belonging to 170 genotypes made up the one very large clonal complex with 10 out of the 11 genotypes of strains isolated from industrial products being found within this group. The overall *P. aeruginosa* eBURST population structure was similar to that of Cramer *et al.* (Cramer N. *et al.* 2012), depicting a population where recombination, rather than point mutation, is the major force driving evolution (Turner *et al.* 2007). The eBURST analysis also illustrated that the population was represented by a few dominant abundant clones (shown by the larger circles in Figure 5.2), and multiple rarer genotypes. There were overlaps between genotypes isolated from different environments (Cramer N. *et al.* 2012), although this is not shown on Figure 5.2 as the isolation sources are not elaborated. This diverse spread of strains was also the case for strains isolated from industrial products, as 1) 4/11 (36%) of the genotypes were novel, and 2) the remaining 7 industrial genotypes had been associated previously with varied habitats, for example cystic fibrosis patients, clinical settings and the natural environment, and previously documented *P. aeruginosa* clone types (Clones Y, D, B and X) (Table 5.2).

Table 5.2 ArrayTube (AT) genotypes of *Pseudomonas aeruginosa* strains

Strain (RW#)	Other strain designations	Isolation source/comment	16 digit code	Hexadecimal code (AT-genotype)	Database match and comments
11	NCTC 12903; ATCC 27853	CLIN; Antibiotic efficacy testing reference strain; originally isolated from blood	0001-1011-1010-1010	1BAA	Novel type
18		CLIN; Chronic prostatitis isolate	1101-0100-0010-0001	D421	Clone A (PA14), previously associated with CF,CLIN, COPD, KER, ENV
27	A128	CF; (Mahenthiralingam *et al.* 1996)	1110-1000-0100-1010	E84A	Clone A12, previously associated with CF, CLIN
30	A128a	CF; (Mahenthiralingam *et al.* 1996)	1110-1000-0100-1010	E84A	Clone A12, previously associated with CF, CLIN
99	MB2.2	ENV; Domestic isolate, washing machine drawer biofilm	1110-1010-0000-1010	EA0A	Clone A3, previously associated with CF, CLIN, COPD, KER, ENV
109*		IND; Strain used in preservative efficacy testing	0110-1100-0010-0010	6C22	Clone Y, previously associated with CF, ENV
110*		IND; Strain used in preservative efficacy testing	1111-0100-0110-1001	F469	Clone D, previously associated with CF, CLIN, COPD, KER, ENV
130*		IND; DWL; Italy; isolated 2010 (Italy RAPD type 1)	1010 -1111-1010-1010	AFAA	Novel type
131*		IND; DWL; Italy; isolated 2010 (Italy RAPD type 2)	1010 -1111-1010-1010	AFAA	Novel type
138*		IND; LAC; Italy; isolated 2001(Italy RAPD type 3)	0110-1101-1001-0010	6D92	Clone H, previously associated with CF, CLIN, COPD, ENV
146*		IND; DWL; Italy; isolated 2004 (Italy RAPD type 2)	1010 -1111-1010-1010	AFAA	Novel type
149*		IND; PC; origin location unknown; isolated 2003	0010-1100-0101-0010	2C52	Novel type
151*	ATCC 9027	CLIN; reference strain used in industrial testing; originally isolated from an outer ear infection (Italy RAPD type 2)	0101-1100-0001-1010	5C1A	Novel type
168.2*		IND; FWS029; typing unsuccessful	-	-	-
172*		IND; DWL; suspected origin is Thailand; isolated 2009	1110-0100-0010-1001	E429	Clone B, previously associated with CF, CLIN, COPD, KER, ENV
176*		IND; DWL; Indonesia; isolated 2010	0010-1100-0101-0010	2C52	Novel type
184*		IND; DWL; UK; isolated 2006	0001-1011-1010-1010	1BAA	Novel type
192*		IND; SC; isolated from a contaminated product whilst on placement at Unilever 2012	0110-1100-0010-0010	6C22	Clone Y, previously associated with CF, ENV
196*	ATCC 13388; NCTC 8060	Origin unknown; reference strain used in industrial testing (ISO 846C)	0000-1011-1001-0010	0B92	Clone X, previously associated with ENV
198*	ATCC 15442	ENV; reference strain used in industrial testing, originally isolated from an animal room water bottle	0000-0101-1001-1010	059A	Genotype associated with CF
199*		IND; MWF	0010-1111-1010-1010	2FAA	Genotype associated with CF and ENV
200*		IND; TC	0010-1011-1001-0010	2B92	Novel type
202*		IND; LAC; EU; isolated 2012	1011-0100-0110-1001	B469	Genotype associated with KER
204*		IND; DWL; isolated 2012	0010-1100-1001-1010	2C9A	Genotype associated with ENV

Abbreviations: CLIN, clinical; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; KER, keratitis; ENV, environmental; IND, industrial; DWL, dish wash liquid; LAC, liquid abrasive cleaner; PC, personal care product; FWS, fabric wash; SC, surface cleaner; MWF, metal working fluid; TC, timber care; RAPD, random amplified polymorphic DNA typing. Genotypes highlighted in blue are strains isolated from the same geographic location at different dates, with the exception of RW#151 which is a reference strain used in 'period after opening' studies at Unilever. The RAPD types of these 5 strains have been detailed in order to investigate concordance between RAPD and AT genotyping methods.

*AT-genotypes for these strains (as strains associated with industry) are highlighted in Figure 5.2

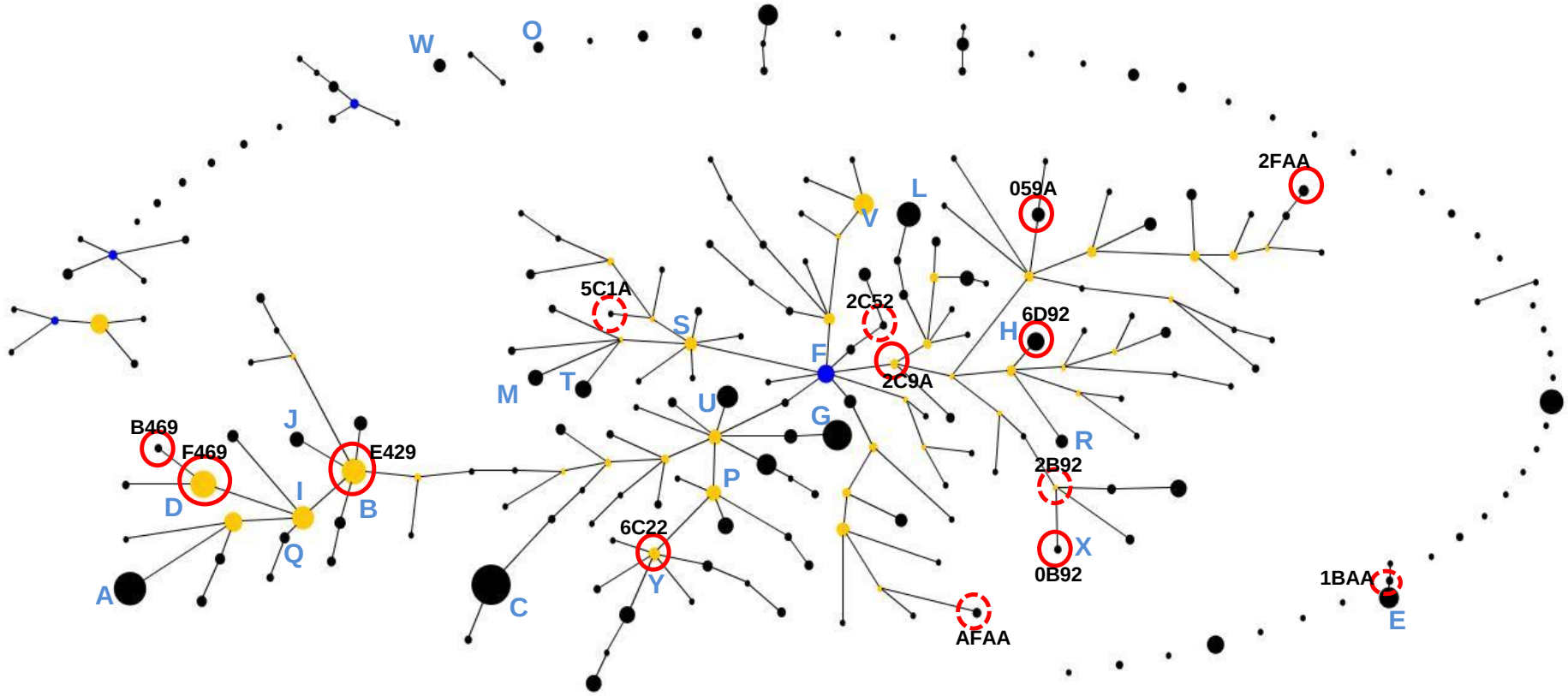


Figure 5.2 Distribution of industrial strains in the *P. aeruginosa* population. The clonal complex structures of 940 strains from individuals with cystic fibrosis, other human infections, environmental sources and industry (n=15) are shown, in addition to reference strains used in industrial testing but initially isolated from clinical (n=1; 5C1A), environmental (n=1; 059A) and unknown sources (n=1; 0B92) . Clonal complexes were calculated from the 16-marker AT genotype of the core genome by the eBURST algorithm. AT-genotypes are indicated by the black, blue and yellow circles, with the diameter of the circle being proportional to the number of isolates with this genotype. Founding AT-genotypes are shown in blue, subfounders in yellow and prevalent clones are marked with light blue letters next to their corresponding AT-genotype. The AT-genotypes that the 18 industry-associated strains belong to are marked with closed (previously reported AT-genotypes) and broken (novel AT-genotypes) red circular outlines. Four letter AT-genotypes are indicated in black next to the red circular outlines.

5.2.3. Genomic diversity of *P. aeruginosa* strains

To define the population biology of the 24 selected RW strains (Table 5.2) at high resolution, WGS and analysis was performed (see methods section 2.10.3). Using the BIGSdb software platform 44 *P. aeruginosa* strains (Table 5.3), including the 24 genome sequenced strains from the RW collection and a selection of *P. aeruginosa* strains from the PubMLST database, were subjected to MLST, rMLST and wgMLST to investigate overall genomic diversity (Maiden *et al.* 2013).

5.2.3.1. MLST

MLST profiles, based on the scheme of Curran *et al.* (Curran *et al.* 2004), were generated for all 44 *P. aeruginosa* strains, resulting in a total of 32 seven-locus sequence types (STs), of which 6 were novel (strains 2192, C3719, MTB-1, RW110/RW202, RW130/RW131/RW146, and RW168.2)(Table 5.3). New alleles were found at the *gua* (strains 2192 and C3719) and *mut* loci (strains 2192 and 168.2). Only 3 strains were assigned to clonal complexes by the BIGSdb analysis, PAO1 (PAO1 clonal complex), UCBPP-PA14 and RW18 (both PA14 clonal complex). The industrial isolates were represented by 10 STs, which were a mixture of previously found (7 STs) and novel STs (3 STs). Searches of the PubMLST databases indicated that STs of the majority of strains, including those of industrial origin, have been associated with multiple environments (Table 5.3). Two exceptions were found, both of which were strains of cystic fibrosis origin (LESB58 and LES431), and members of the Liverpool epidemic strain type (Fothergill *et al.* 2012). The phylogenetic network produced from the 44 MLST profiles shown in Figure 5.3 illustrates the diversity of the *P. aeruginosa* strains that were investigated. Aside from the clustering of LESB58 and LES431, strains of diverse origin were distributed fairly evenly throughout the network, suggesting no association between ST and isolation source (Figure 5.3).

5.2.3.2. rMLST

All 53 ribosome protein subunits (*rps* genes) were identified in 42 out of the 44 *P. aeruginosa* strains allowing rMLST analysis (Jolley *et al.* 2012a) to be performed. The strains 2192 and C3719 were excluded from further analysis as they had multiple missing or truncated *rps* loci within their rMLST profiles. A total of 7 new *rps* alleles (strain VRFPA04 at the BACT000020, BACT000053 and BACT000057 loci; RW168.2 at the BACT000001 and BACT000007 loci; RW204 at the BACT000040 locus, and; NCGM_1984 and NCGM_1900 at the BACT000012 locus)

and 36 unique ribosomal MLST profiles (rSTs) were identified from the 42 complete rMLST profiles. Strains sharing the same rST were RW130/RW131/R146, RW110/RW202, RW109/RW192, RW27/RW30 and NCGM_1984/NCGM_1900. A greater number of unique STs was observed for rMLST (n=36), which profiles 53 loci, than MLST (n=32), which profiles only 7 loci (Curran *et al.* 2004). The NeighbourNet phylogeny based on the complete rMLST profiles of the 42 *P. aeruginosa* strains (Figure 5.4), however, showed good congruence with the MLST NeighbourNet phylogeny (Figure 5.3). Strains were grouped into the same clusters with both approaches, although resolution at the nodes was improved for the rMLST profiles. Clusters included: RW130, RW131 and RW 146; ATCC 12903 and RW184; RW27 and RW30; LESB58 and LES431; RW109, RW138 and RW192; RW149 and RW176; RW18 and UCBPP-PA14; RW110 and RW202, and; NCGM_1984, NCGM_1900 and NCGM2.S1.

5.2.3.3. wgMLST

wgMLST (Maiden *et al.* 2013)was performed with the 44 *P. aeruginosa* strains using the genome sequence of *P. aeruginosa* UCBPP-PA14 as a comparator for analysis. Out of the 5883 loci identified in the *P. aeruginosa* UCBPP-PA14 reference genome, 4579 were shared between all *P. aeruginosa* strains. A NeighbourNet phylogeny generated by pairwise comparisons of the 4579 shared loci is shown in Figure 5.5. The degree of reticulation seen in the wgMLST NeighbourNet plot (Figure 5.5) is greatly reduced compared to the MLST and rMLST NeighbourNet plots (Figures 5.3 and 5.4, respectively), illustrating the increased resolution afforded by including a higher number of loci in the analysis. Although each strain was identified as a unique ST by wgMLST (n=44 STs), there was concordance between the clusters of strains found by all three MLST-based methods. The same strain groupings were found as those outlined in section 5.3.3.2 for the rMLST scheme. The NeighbourNet plot topology suggested the divergence of a lineage containing RW110, RW202, NCGM_1984, NCGM_1900, NCGM2.S1, RW172, MTB-1, UCBPP-PA14, RW18, VRFPA04, B136-33 and PA7 from the larger strain subgroup. To clarify this, more strains would need to be included in the analysis.

Table 5.3 MLST allele and Sequence Type (ST) designations for the 44 *P. aeruginosa* strains

***P. aeruginosa* strain**	**Isolation source**	**MLST loci**							**ST**	**ST isolation sources[a]**	**clonal complex[a] (AT-type clone)[b]**
		acs	*aro*	*gua*	*mut*	*nuo*	*pps*	*trp*			
LESB58	CF	6	5	11	3	4	23	1	146	CF	(Clone T)
PAO1	CLIN	7	5	12	3	4	1	7	549	*	PA01 (Clone W)
UCBPP-PA14	CLIN	4	4	16	12	1	6	3	253	CF, CLIN, ENV, OTHER	PA14 (Clone A)
PA7	CLIN	87	34	43	37	53	107	126	1195	OTHER	
2192	CF	6	6	-	-	3	4	7	Novel 1		
C3719	CF	28	5	-	18	4	13	3	Novel 2		(Clone O)
PACS2	CF	11	5	6	3	74	13	7	1394	*	
B136-33	CLIN	2	4	24	3	1	6	25	1024	*	
DK2	CF	17	5	11	18	4	10	3	386	CF, SPUTUM	(Clone A2)
LES431	CF	6	5	11	3	4	23	1	146	CF	(Clone T)
M18	ENV	16	5	1	3	4	15	7	1239	OTHER	
MTB-1	ENV	5	8	3	5	1	11	3	Novel 3		
NCGM2.S1	CLIN	38	11	3	13	1	2	4	235	CF, CLIN, ENV, OTHER	
PA1	ENV	15	3	3	11	1	15	1	782	CF, OTHER	
RP73	CF	11	5	11	11	3	27	7	198	CF, CLIN	
SCV20265	CF	17	5	36	3	3	7	3	299	CF, OTHER	
VRFPA04	CLIN	32	13	24	13	1	6	25	823	*	
F22031	CLIN	11	76	5	3	61	14	3	485	CF	
NCGM_1984	CLIN	38	11	3	13	1	2	4	235	CF, CLIN, ENV, OTHER	
NCGM_1900	CLIN	38	11	3	13	1	2	4	235	CF, CLIN, ENV, OTHER	
RW 11; NCTC_12903	CLIN	28	5	36	3	3	13	7	155	CF, ENV, OTHER	
RW18	CLIN	4	4	16	12	1	6	3	253	CF, CLIN, ENV, OTHER	PA14 (Clone A)
RW27	CF	6	5	6	34	27	3	7	162	CF, CLIN	(Clone A12)
RW30	CF	6	5	6	34	27	3	7	162	CF, CLIN	(Clone A12)
RW99	ENV	6	5	6	7	4	6	7	27	CF, CLIN, ENV, OTHER	(Clone A3)
RW109	IND	17	5	5	4	4	4	3	111	CF, CLIN, ENV, OTHER	(Clone Y)
RW110	IND	5	141	65	151	1	33	50	Novel 4		(Clone D)
RW130	IND	15	48	20	142	4	7	7	Novel 5		
RW131	IND	15	48	20	142	4	7	7	Novel 5		
RW138	IND	17	5	5	4	4	4	3	111	CF, CLIN, ENV, OTHER	
RW146	IND	15	48	20	142	4	7	7	Novel 5		
RW149	IND	1	5	26	3	1	10	3	1342	OTHER	
RW151; ATCC 9027	CLIN	23	5	12	30	1	4	7	1105	CF	
RW168.2	IND	40	5	3	-	73	75	2	Novel 6		

RW172	IND	13	8	9	3	1	6	9	316	CF, CLIN, ENV, OTHER	(Clone B)
RW176	IND	1	5	26	3	1	10	3	1342	OTHER	
RW184	IND	28	5	36	3	3	13	7	155	CF, ENV, OTHER	
RW192	IND	17	5	5	4	4	4	3	111	CF, CLIN, ENV, OTHER	(Clone Y)
RW196; ATCC 13388	UNKNOWN	17	5	12	3	14	4	7	244	CF, CLIN, ENV, OTHER	(Clone X)
RW198; ATCC 15442	ENV	6	28	4	3	3	4	7	252	CF, CLIN, ENV, OTHER	
RW199	IND	17	22	11	3	3	15	3	800	CF	
RW200	IND	6	5	6	5	4	4	7	641	CLIN	
RW202	IND	5	141	65	151	1	33	50	Novel 4		
RW204	IND	6	5	5	3	3	13	1	645	CF, CLIN,ENV	

CLIN, clinical; CF, cystic fibrosis; ENV, environmental; IND, industrial; ' – ' designates a new allele type or ST not within the PubMLST database; [a]Isolation source and clonal complex from PubMLST database; [b]clone type from AT-genotyping and database searches; '*' no information available on PubMLST database for this ST; Cells within the ST column shaded in the same colour correspond to the same MLST profile

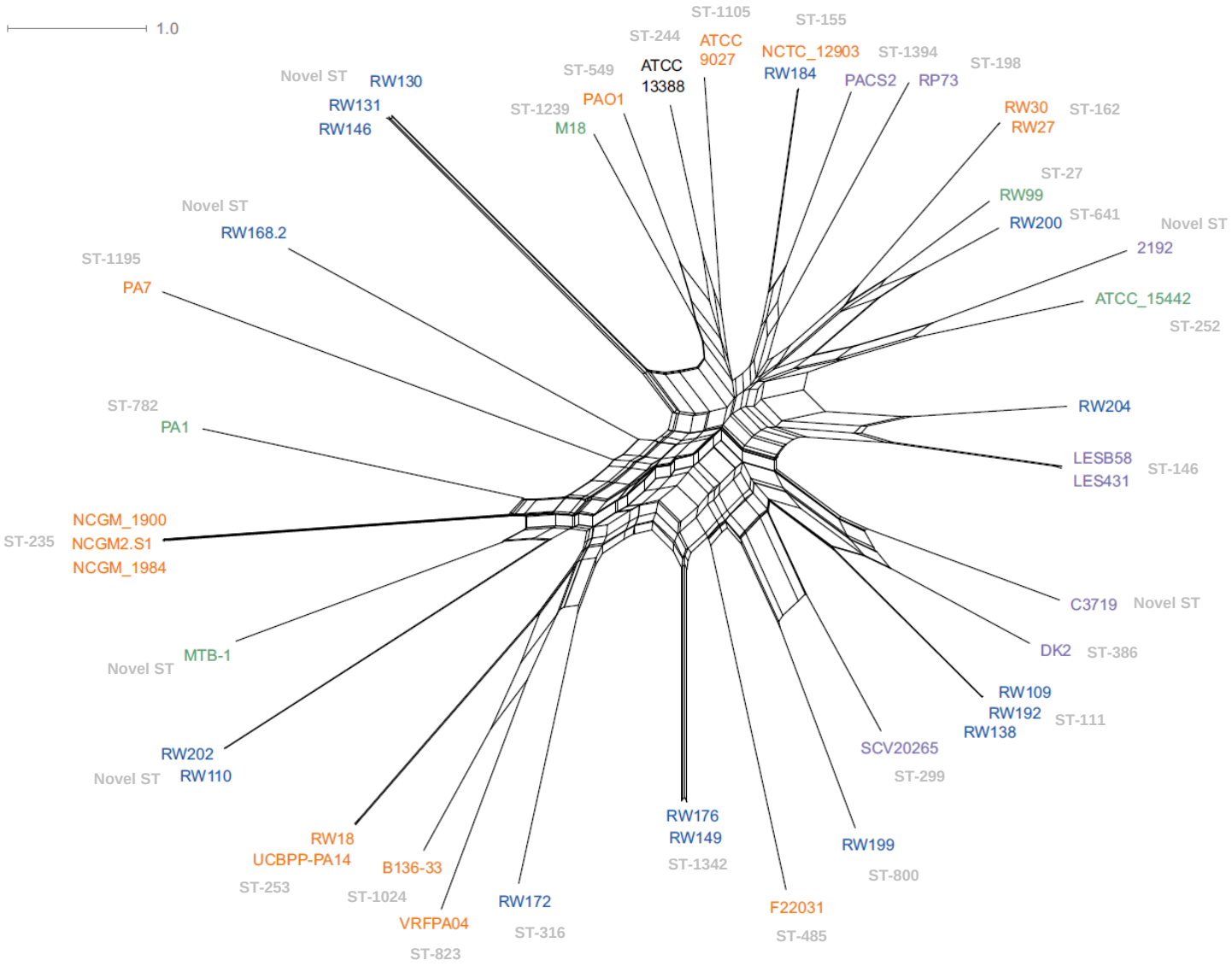


Figure 5.3 Multilocus sequence typing (MLST)-based analysis of *P. aeruginosa* whole genome sequence data. The diversity of the 44 *P. aeruginosa* strains based on the allelic diversity at 7 MLST loci is illustrated. Analyses were carried out using the Genome Comparator tool of the BIGSdb and phylogenetic networks generated using the NeighbourNet algorithm in SplitsTree v4.13.1. Strain names are colour coded to indicate isolate source: purple strains are of cystic fibrosis origin; orange strains are of clinical origin; green strains are of environmental origin; blue strains are of industrial origin, and; black strains are of unknown origin. Sequence types are shown in grey next to strain names on the phylogenetic network. The scale bar represents distances calculated from the number of loci.

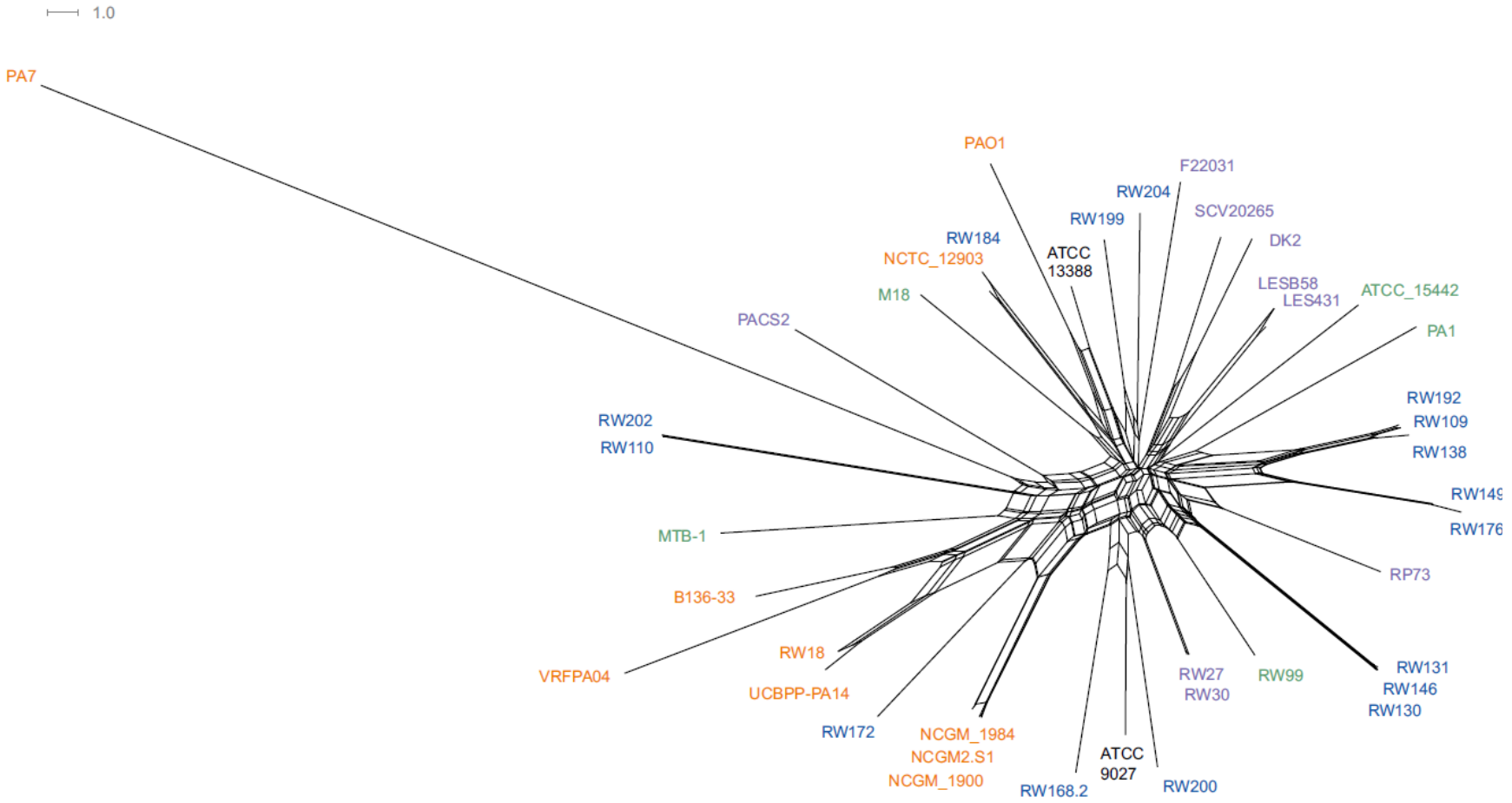


Figure 5.4 Ribosomal multilocus sequence typing (rMLST)-based analysis of *P. aeruginosa* whole genome sequence data. The diversity of 42 *P. aeruginosa* strains based on the allelic diversity at 53 rMLST loci is illustrated. *P. aeruginosa* strains with incomplete rMLST profiles (2192 and C3719) were excluded. Analyses were carried out using the Genome Comparator tool of the BIGSdb and phylogenetic networks generated using the NeighbourNet algorithm in SplitsTree v4.13.1. Strain names are colour coded to indicate isolate source: purple strains are of cystic fibrosis origin; orange strains are of clinical origin; green strains are of environmental origin; blue strains are of industrial origin, and; black strains are of unknown origin. The scale bar represents distances calculated from the number of loci.

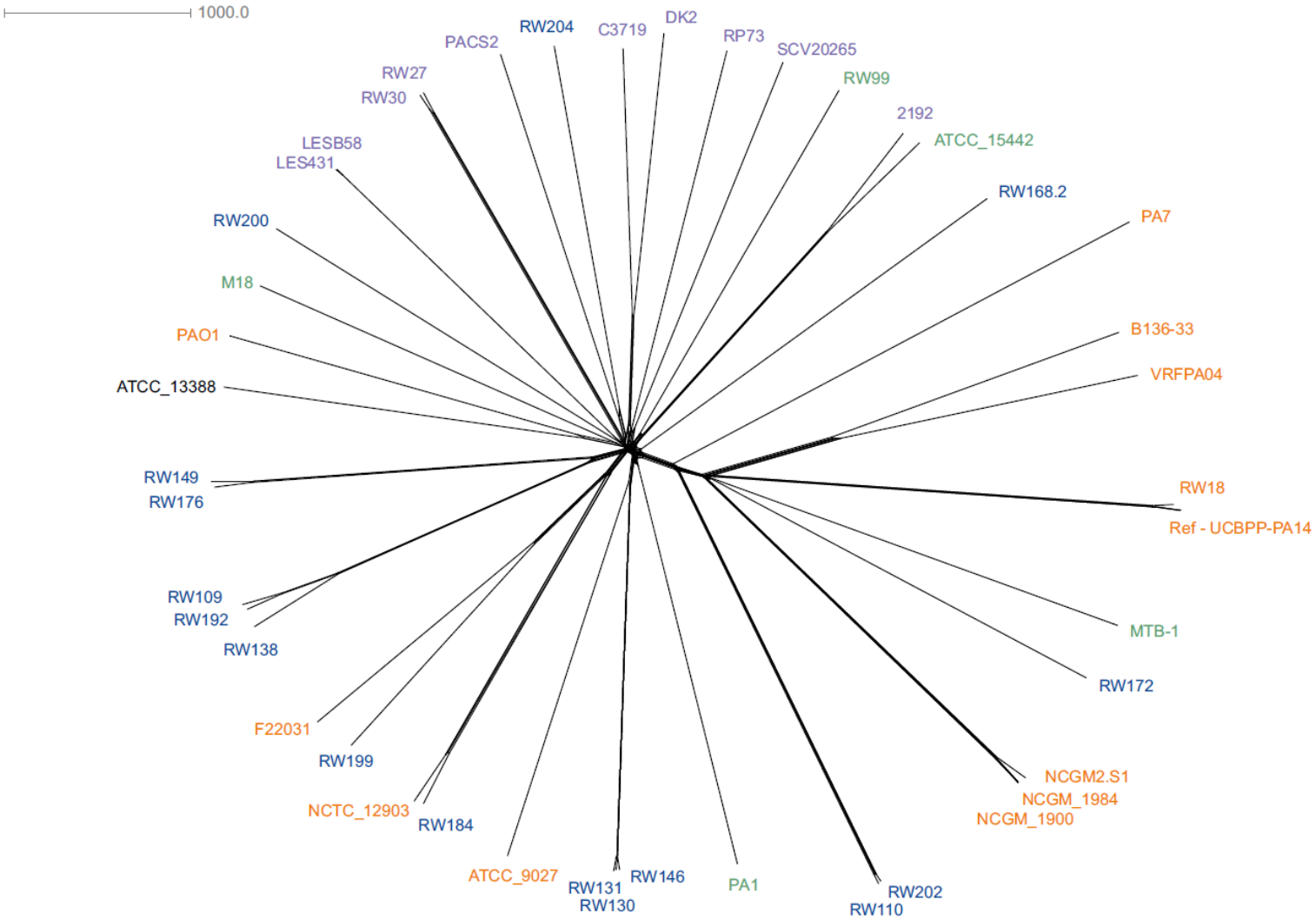


Figure 5.5 Whole genome multilocus sequence typing (wgMLST)-based analysis of *P. aeruginosa* whole genome sequence data. The diversity of the 44 *P. aeruginosa* strains based on the allelic diversity at 4579 loci of a reference genome sequence (*P. aeruginosa* UCBPP-PA14) is illustrated. Analyses were carried out using the Genome Comparator tool of the BIGSdb and phylogenetic networks generated using the NeighbourNet algorithm in SplitsTree v4.13.1. Strain names are colour coded to indicate isolate source: purple strains are of cystic fibrosis origin; orange strains are of clinical origin; green strains are of environmental origin; blue strains are of industrial origin, and; black strains are of unknown origin. The scale bar represents distances calculated from the number of loci.

5.2.4. Congruence between genotyping methods

Broadly, where genotypes were shared between multiple strains there was agreement between the AT-genotypes and the MLST STs (Table 5.4). Exceptions to this were the strains RW109, RW192 and RW138 which shared the MLST genotype ST-111, but were represented by two different AT-genotypes (RW109 and RW192 were 6C22, whilst RW138 was 6D92), and RW110 and RW202 which were both classed as Novel ST-4 by MLST but had different AT-genotypes, F469 and B469 respectively. Between the AT-genotypes 6C22 and 6D92 there were differences in 4/16 single nucleotide polymorphisms (SNPs) of the core genome (C → D, 1 SNP change; 2 → 9, 3 SNP changes), whilst between F469 and B469 there was 1 SNP difference (F → B). As little as 1 SNP change can lead to a change in AT genotype, but it is unknown how many SNP changes correspond to a change in ST as defined by MLST.

There was less congruence between RAPD-PCR types and the AT genotypes or MLST STs. Of the genotype clusters observed for AT-genotyping or MLST (Table 5.4) only two strains within one cluster shared the same RAPD profile (RW131 and RW146). There were also examples where a strain within a cluster had a different RAPD profile to others of the same AT genotype or ST (RW130 within the RW130, RW131 and RW146 cluster, and strains of the RW109, RW192 and RW138 cluster), and where a strain had the same RAPD profile as strains within a cluster, but did not share the AT genotype or ST (RW151 had the same RAPD profile as both RW131 and RW146). RAPD-typing was used only as a preliminary genotyping method aimed at removing multiple highly clonal strains obtained from single sources (e.g. sequential isolates from the same contamination incident), however, and was not used to inform population structure investigations.

Table 5.4 Genotypes associated with the clusters of *P. aeruginosa* strains identified by MLST.

Strain	Isolation source/comment	AT-genotype	MLST*
RW130	IND; DWL; Italy; isolated 2010 (Italy RAPD type 1)	AFAA	Novel ST-5
RW131	IND; DWL; Italy; isolated 2010 (Italy RAPD type 2)		
RW146	IND; DWL; Italy; isolated 2004 (Italy RAPD type 2)		
ATCC 12903	CLIN; Antibiotic efficacy testing reference strain; originally isolated from blood	1BAA	ST-155
RW184	IND; DWL; UK; isolated 2006		
RW27	CF; (Mahenthiralingam *et al.* 1996)	E84A	ST-162
RW30	CF; (Mahenthiralingam *et al.* 1996)		
LESB58	CF	4C12	ST-146
LES431	CF		
RW109	IND; Strain used in preservative efficacy testing	6C22	ST-111
RW192	IND; SC; isolated from a contaminated product whilst on placement at Unilever 2012		
RW138	IND; LAC; Italy; isolated 2001(Italy RAPD type 3)	6D92	
RW149	IND; PC; origin location unknown; isolated 2003	2C52	ST-1342
RW176	IND; DWL; Indonesia; isolated 2010		
RW18	CLIN; Chronic prostatitis isolate	D421	ST-253
UCBPP-PA14	CLIN		
RW110	IND; Strain used in preservative efficacy testing	F469	Novel ST-4
RW202	IND; LAC; EU; isolated 2012	B469	
NCGM_1984	CLIN	-	ST-235
NCGM_1900	CLIN		
NCGM2.S1	CLIN		

Abbreviations: MLST, multi-locus sequence typing; AT-typing, Array-Tube typing; CLIN, clinical; CF, cystic fibrosis; IND, industrial; DWL, dish wash liquid; LAC, liquid abrasive cleaner; PC, personal care product; RAPD, random amplified polymorphic DNA typing. Genotypes highlighted in blue are strains isolated from the same geographic location at different dates

*only STs for the 7-loci MLST scheme are given as clustering of strains within NeighbourNet phylogenies for MLST, rMLST and wgMLST resulted in the same strain groups; '–' AT-genotype unknown

5.3. Discussion

Strain typing techniques are not routinely applied to industrial contaminants, and as such the diversity of bacterial strains in industrial settings is unknown. In addition, no population biology studies of *P. aeruginosa* have included isolates of industrial origin, having mainly focussed on clinically relevant strains and, to a lesser extent, environmental strains. The population structure of *P. aeruginosa* is thought to be non-clonal epidemic, with clinical isolates being indistinguishable from environmental isolates in terms of genotypic, taxonomic and metabolic characteristics (Morales *et al.* 2004; Pirnay *et al.* 2009; Cramer N. *et al.* 2012). This chapter genotyped a collection of clinical, environmental and industrial *P. aeruginosa* isolates to determine if specific strains/dominant clones were associated with industrial contamination, and if these strains were genetically different to those encountered in clinical and natural environments.

5.3.1. Strain diversity and population biology of *P. aeruginosa* in industry

To gain insight into the strain diversity associated with contaminated HPC products, a collection of 69 industrial *P. aeruginosa* isolates were subjected to RAPD-PCR typing, leading to the identification of 26 different strain types. No firm conclusions could be drawn linking strain type to product type or isolation location due to limitations with the dataset, but there was overlap between strain types and product types suggesting no selection for habitat; when overlaps in RAPD strain type were observed these were primarily linked to isolates from the same contamination incident. RAPD typing facilitated the selection of industrial *P. aeruginosa* strains for further genotypic analyses; 19 isolates from the industrial collection were chosen on the basis of the RAPD typing.

To investigate *P. aeruginosa* population biology at greater resolution more robust strain typing techniques were used. Twenty-four *P. aeruginosa* strains, 19 of which were associated with industry, were subjected to AT-typing and WGS for MLST-based analyses. AT types were obtained for 23 strains and compared to 917 other *P. aeruginosa* strains held within a previously established database (Cramer N. *et al.* 2012). This revealed that 4/11 of the genotypes were novel, with the remaining types being previously found and linked to diverse environments. The AT genotypes were also mainly spread throughout the largest clonal complex of the eBURST diagram suggesting that they were more related to each other than they were to genotypes isolated from other environments. Even though the industrial strain panel

was small (<5% total strains within the AT-typing database), the genotype distribution corroborated the current hypothesis that isolates from different environments are genotypically highly similar to each other. Due to the limited numbers of the industrial strain panel, the topology of the eBURST diagram was not dramatically changed from that of Cramer *et al.* (Cramer N. *et al.* 2012) and indicated a non-clonal epidemic population structure. To comprehensively assess *P. aeruginosa* population structure, future investigations need large numbers of similar proportions of strains from different environments, including industry.

The sequence-based typing techniques MLST, rMLST and wgMLST (Maiden *et al.* 2013) were also used to genotype the 24 strains from the AT-typing investigations, in addition to a further 20 *P. aeruginosa* strains from the PubMLST database representing diverse isolation sources. The results of the seven-locus MLST indicated that, similarly to the AT-typing analysis, industrial strains belonged to a mixture of previously documented (7/10) and novel STs (3/10), with the previously found STs being associated with multiple habitats. In addition, all of the previously found STs from the panel (Table 5.3) had been found in a variety of environments with the exception of the two cystic fibrosis-linked strains LESB58 and LES431. There is other evidence, however, to suggest these strains are not restricted to cystic fibrosis patients; the LES has caused respiratory infections in parents of a non-CF patient (McCallum *et al.* 2002), and has also been associated with transmission to a pet cat (Mohan *et al.* 2008), although for both of these studies the primary source of the infecting strain was a CF individual.

The NeighbourNet plots of the MLST, rMLST and wgMLST data illustrated that the novel industrial STs did not cluster together, but were diverse. In addition, whilst the resolution was improved as an increasing number of loci were incorporated in the MLST schemes, the same strain clusters were identified for all three typing techniques. The clustering of certain strains was expected, for example for RW130, RW131 and RW146 (DWL strains from Italy, two of which shared the same RAPD profile), but not expected for others. For example, although other industrial strains did cluster together, the strains were of different industrial origins (RW109/RW192/RW138, RW149/RW176 and RW110/RW202) and one cluster comprised strains of clinical and industrial origin (ATCC 12903 and RW184). Overall there was no clear link between genotype and isolation source.

An interesting observation from the NeighbourNet plot of the wgMLST comparison was the divergence of a subgroup of strains from a larger 'core' group (Figure 5.5), which was not apparent from the MLST or rMLST analysis. A recent paper by Stewart *et al.* (Stewart *et al.* 2014) used core genome SNP analysis to compare the genomes of 55 *P. aeruginosa* strains, and had similar findings. The analysis identified two major subgroups and a third smaller outlier

group containing only two isolates, which included PA7. Strains shared between Stewart *et al.* and this PhD study fell into the same subgroups: Group 1 shared strains included PACS2, PAO1, C3719, DK2, M18 and LESB58; Group 2 shared strains included B136-33, NCGM2S1 and PA14. In this chapter, *P. aeruginosa* PA7, RW202 and RW110 fell outside of the two major groups. As concluded by Stewart *et al.*, it is unknown whether strains from Group 1 are actually more abundant than those from Group 2. It is a possibility that a bias in strain selection could have resulted in the NeighbourNet plot topology, and the inclusion of more strains would populate the branches.

5.3.2. Genotyping methods for the investigation of *P. aeruginosa* population biology

Five genotyping methods were investigated during this chapter to assess strain diversity, belonging to three categories: 1) PCR fingerprinting-based methods; 2) micro probe array-based methods, and; 3) sequence-based methods. For *P. aeruginosa*, RAPD-typing, AT-typing and seven-locus MLST have previously been used, with AT-typing and MLST being used most extensively in population biology investigations (Table 5.1). These methods either sample the whole genome (RAPD) or variation within the core genome (AT-typing, seven-locus MLST). Whilst AT-typing and MLST have previously been shown to demonstrate good concordance for *P. aeruginosa*, RAPD typing has higher discriminatory power and is sensitive to genomic rearrangements, which may decrease its utility (Hall *et al.* 2013). In this chapter, RAPD typing was used as a preliminary screen to select strains for further investigations. It was found that, similarly to Hall *et al.* (Hall *et al.* 2013), there was not complete congruence between RAPD and the other typing methods, but AT-typing and seven-locus MLST genotypes were generally concordant.

With the advent of rapid, high-throughput sequencing methods which are becoming increasingly cost effective, it is possible to generate accurate WGS data for bacterial isolates (Maiden *et al.* 2013). Genotyping schemes can take advantage of this data to provide high resolution typing of isolates. The gene-by-gene approach to typing, which is an extension of the MLST scheme, is as a versatile means of examining variation between genome sequences (Maiden *et al.* 2013). MLST analyses are scalable to include few or many genes and may be tailored to the species under investigation. In this chapter, WGS data of *P. aeruginosa* strains was used for MLST, rMLST and wgMLST using BIGSdb (Jolley and Maiden 2010). Increasing the number of loci from 7 (MLST), to 53 (rMLST), to 4579 (wgMLST) resulted in the same strains clustering together in the NeighbourNet plots, but greatly increased the resolution of typing though an increasingly genome-wide analysis. Although only performed for AT-typing in this chapter, eBURST analysis can be applied to other MLST datasets to identify clonal complexes or

lineages and investigate population structure (Feil *et al.* 2004). Extended MLST typing schemes utilising the gene-by-gene approach could therefore be analysed using eBURST.

There are limitations to the gene-by-gene approach however, including that only loci which have been defined will be identified and that loci split over more than one contig of the genome assemblies will not be found by BIGSdb (Sheppard *et al.* 2012). These problems may be negated as assembly and annotation of genomes improves. One further issue with wgMLST is that a reference sequence is required; this will dictate the genes that are included in the analysis and potentially divide the comparator population into groups which are similar or dissimilar to the reference sequence (Sheppard *et al.* 2012). Whilst less user-friendly than BIGSdb, other computational methods are available to compare the full genetic content of bacterial genomes. One such method is the large-scale blast score ratio (LS-BSR) pipeline, which does not use a reference sequence and can identify and compare coding sequences in a set of bacterial genomes (Sahl *et al.* 2014). Further investigations with software such as the LS-BSR pipeline could be performed to gain insight into the pangenome of the *P. aeruginosa* strains examined in this study and potentially identify any industrial-isolate specific genes that were absent in the core genome.

5.3.3. The utility of strain typing methods in industry

Bacterial strain typing techniques are very important in epidemiological investigations, having the ability to link strain types to an outbreak of disease locally or globally, trace sources of infection and monitor the success of treatment for a bacterial infection (Spratt and Maiden 1999). In industry the use of strain typing techniques is rare, but has been applied to bacterial contaminants of HPC products; repetitive element PCR and PFGE were employed to link *B. cenocepacia* strains from a contaminated mouthwash to human infection (Kutty *et al.* 2007). Another industry-related case, involving human infection from the consumption of contaminated turkey products, used PFGE to link a strain of *Listeria monocytogenes* from a food-processing plant to the outbreak (Olsen *et al.* 2005). The outbreak strain also shared the same PFGE profile as a *L. monocytogenes* strain identified in the plant 12 years earlier, suggesting persistence of the strain leading to recurrent product contamination (Olsen *et al.* 2005).

In this chapter, three strains, which were known DWL contaminants from Italy but collected 6 years apart (RW130/131 and RW146), clustered together as the same AT-type and ST. It is therefore likely that a persistent strain is present in the manufacturing environment in Italy. This information could be useful for informing industry if cleaning and decontamination practices have been successful and identify problematic strains. In addition, sampling of factory

pipes and other surfaces, as well as product samples, would allow the identification of potential sources of contamination. Furthermore, as *P. aeruginosa* is hypothesised to be a predominantly aquatic microorganism (Selezska *et al.* 2012) and water is an important raw material in HPC products (Jimenez 2007), factory water supplies should also be monitored.

5.4. Conclusions

The main conclusions from the work in this chapter were as follows:

1) The *P. aeruginosa* strains associated with HPC products were found, by RAPD-PCR, to be a diverse group and there were no links between strain type and product type or location of isolation.

2) Industrial strains belonged to a mixture of previously found and novel AT-types and STs. The previously found genotypes were associated with a plethora of different environments suggesting *P. aeruginosa* displays no selection for habitat.

3) Incorporation of the industrial strains into a large database of *P. aeruginosa* AT-types, followed by eBURST analysis, confirmed the non-clonal epidemic population structure of *P. aeruginosa.*

The hypothesis proposed at the beginning of this chapter was accepted:

Hypothesis: *P. aeruginosa* has a non-clonal epidemic population structure and strains isolated from industrial settings share genotypes with strains of cystic fibrosis, clinical and environmental origin.

6 Preservative susceptibility and phenotypic characterisation of an industrial *P. aeruginosa* strain panel

The growth dynamics and motility data for strains belonging to the international *Pseudomonas aeruginosa* reference panel (De Soyza *et al.* 2013) were part of the following manuscript:

Cullen, L., Weiser, R., Olszak, T., Maldonado, R., Moreira, A. S. *et al.* (2015). Phenotypic characterisation of an international *P. aeruginosa* reference panel: Strains of cystic fibrosis origin show less *in vivo* virulence than non-CF strains. *Microbiology* **161**(10):1961-1977.

6.1 Introduction

The resistance of *P. aeruginosa* to antibiotics is very well documented, and contributes to its success as an opportunistic pathogen (Poole 2011). Treatment of *P. aeruginosa* is hampered by high levels of antibiotic resistance conferred via intrinsic, adaptive, or acquired mechanisms, many of which we have a good understanding of (Tenover 2006). In comparison, relatively little is known about the susceptibility of *P. aeruginosa* to biocides. Similarly, the modes of action of biocides and the mechanisms involved in resistance are poorly understood, complicated by the multitude of cellular targets that are affected (Maillard 2002).

In general, antimicrobial activity differs greatly between different groups of micro-organisms and even between strains of the same species (Maillard 2002). This has been demonstrated in recent publications looking at the biocide susceptibilities of another group of problematic industrial contaminants, the *Burkholderia cepacia* complex bacteria (Rose *et al.* 2009; Rushton *et al.* 2013). In these studies large and diverse strain collections representing multiple species were investigated and found to be highly variable in their susceptibilities to biocides and preservatives. Another recent study examined *Enterobacter gergoviae* as an industrial contaminant (Périamé *et al.* 2014) and found that across a panel of 12 strains, susceptibilities were both variable and non-variable for the different preservatives tested; for four out of the eight agents tested the minimum inhibitory concentrations were the same across the strain panel. For *P. aeruginosa,* analyses of both antibiotic and biocide susceptibility have found variable levels of antimicrobial tolerance (Van Eldere 2003; Lambert 2004). These analyses did not consider strains isolated from industry, however, and did not investigate commonly used HPC preservatives such as parabens, formaldehyde releasing agents, isothiazolinones and phenoxyethanol. Of the two studies which have included industrial strains of *P. aeruginosa*, one

was performed over a decade ago and did not report absolute minimum inhibitory concentration values for the preservatives tested (Chapman *et al.* 1998), and the other investigated only quaternary ammonium compounds as biocides, finding that clinical strains were the most tolerant and industrial strains were the most sensitive (Lambert *et al.* 2001). Further surveys of the biocide susceptibility of *P. aeruginosa* would be useful to understand the presence of *P. aeruginosa* in environments of frequent biocide exposure. As a common contaminant of HPC products, it would be interesting to determine whether *P. aeruginosa* has a high tolerance for preservatives used in industry.

Preservatives are used in industry to prevent spoilage of products, and preservation strategies are complex (Orth *et al.* 2006)(see section 1.1.4). Preservative enhancing agents are often incorporated into formulations to improve preservative efficacy. These compounds are not classed as preservatives, nor regulated under the same legislation, and alone do not necessarily have an antimicrobial effect (Varvaresou *et al.* 2009). Examples include the chelating agent EDTA and the surfactant-like substances caprylyl glycol and ethylhexylglycerin (Varvaresou *et al.* 2009). Combinations of preservatives and enhancers can extend the spectrum of antimicrobial activity, lower the levels of individual preservatives and therefore risks of toxicity to the consumer, and may exhibit an additive or synergistic effect (Orth *et al.* 2006).

In the context of preservatives, synergy is a greater than expected combined antimicrobial effect than would be observed from the simple addition of the activities of the individual agents (Denyer *et al.* 1985). Incidences where synergy has been observed between preservatives and exploited in industry include phenoxyethanol and parabens (Phenonip) and monolaurate and sorbic acid (Lauribic) (Denyer *et al.* 1985). As the majority of in-use preservatives belong to three classes (parabens, formaldehyde-releasing agents and isothiazolinones) and very few new preservatives have been approved in recent years (Lundov *et al.* 2009), the discovery of novel additive or synergistic combinations of available preservatives, or effective preservative enhancers is of great interest to industry. In addition, the use of lower concentrations of preservatives and milder preservation systems has become increasingly important to the consumer (Varvaresou *et al.* 2009). Microorganisms that overcome preservation strategies to cause contamination must possess a variety of resistance mechanisms and be capable of adapting to the harsh conditions created by the different preservatives and preservative enhancing agents used in HPC product formulations.

At present, there is no published literature on the phenotypic characterisation of *P. aeruginosa* strains isolated from industrial sources such as HPC products. Previous studies including both clinical and environmental settings have determined *P. aeruginosa* populations to be freely

recombining but punctuated with epidemic clones, and that there is little association between genotype and habitat (Kidd *et al.* 2012). In general, it is also thought that strains from varying environments are functionally indistinguishable from one another (Römling *et al.* 1994; Rahme *et al.* 1995; Foght *et al.* 1996; Alonso *et al.* 1999), but as mentioned previously, these studies have not considered industry as a source. Chapter 5 examined a collection of *P. aeruginosa* isolates from HPC contamination incidents, in addition to reference isolates used in industrial testing, to gain insight into the diversity present. The findings of these investigations largely corroborated the consensus model of *P. aeruginosa* population structure, but gave no indication of the phenotypic characteristics of the isolates.

In this chapter, a panel of 40 *P. aeruginosa* strains, including a selection from the genetically characterised industrial collection, and from the recently published international *P. aeruginosa* reference panel (De Soyza *et al.* 2013) were examined. The 40 strains derived from industrial, cystic fibrosis, clinical, environmental and unknown sources and were subjected to preservative susceptibility assays for a number of commonly used HPC preservatives, in addition to preservative-preservative and preservative-preservative enhancer combinations of industrial interest. Growth curve and motility assays were also performed to elucidate any other phenotypic differences between the strains across isolation source. Furthermore, the development of adaptive resistance to a preservative combination was examined using planktonic growth and biofilm experimental evolution models.

6.1.1 Aims

The aims of this chapter were as follows:

1) Determine the susceptibility of a panel of 40 *P. aeruginosa* strains to preservatives commonly used in the HPC industry

2) Evaluate the efficacy of novel preservative-preservative and preservative-preservative enhancing agent combinations using a sub-set of the *P. aeruginosa* panel strains, in an attempt to identify combinations with elevated anti-*P. aeruginosa* activity

3) Investigate the growth dynamics in liquid media and the swimming, swarming and twitching motilities of the panel of 40 *P. aeruginosa* strains

4) Determine whether differences in preservative susceptibility profiles or other phenotypic characteristics were linked to a clinical, environmental or industrial isolation source

5) Examine the evolution of *P. aeruginosa* preservative resistance using both planktonic growth and biofilm growth models

Hypothesis 1: The phenotypic characteristics of *P. aeruginosa* strains are highly variable and unlinked to habitat

Hypothesis 2: Adaptive resistance can be developed to a preservative combination in planktonic and biofilm growth models, where *P. aeruginosa* can grow in preservative concentrations above the MIC level of the ancestral strain.

6.2 Results

6.2.1 Preservative susceptibility

6.2.1.1 Individual preservatives

A broth dilution assay was used to determine the MICs of 6 preservatives for a collection of 40 *P. aeruginosa* strains from cystic fibrosis (CF; n=10), clinical (CLIN; n=9), environmental (ENV; n=5), industrial (IND; n=15) and unknown (n=1) sources. The preservatives that were tested belonged to four different classes: isothiazolinones (methylisothiazolinone, MIT; chloromethylisothiazolinone and methylisothiazolinone blend in the ratio 3:1, CITMIT; benzisothiazolinone, BIT); alcohols (phenoxyethanol, PHE); biguanides (chlorhexidine, CHX), and; organic acids (benzoic acid, BA). For each strain, 4 replicates were recorded and the median and range of the data recorded in Table 6.1 (MIT, CITMIT and BIT) and Table 6.2 (PHE, CHX and BA). The susceptibility of *P. aeruginosa* for each preservative varied considerably across the strain panel, illustrated in Figure 6.1.

The MIC data for MIT, CITMIT, BIT and CHX were more widely distributed than for PHE and BA, where the growth of the majority of strains was inhibited at one MIC value, 0.46875% (25/40 strains) and 0.05% (35/40 strains) respectively (Table 6.1). Strains were identified which had high MIC values for more than one preservative. For example RW138 had the highest recorded MIC for MIT (0.005%), BIT (0.04%) and PHE (1.25%), RW176 had the highest recorded MIC for MIT (0.005%), CITMIT (0.005%) and the second highest MIC for BIT (0.02%), and RW192 had the highest recorded MIC for BIT (0.04%) and the second highest recorded MIC for MIT (0.00375%). Strains were also identified with low MICs for multiple preservatives, for example Pa#1, Pa#4, Pa#7 and Pa#8 were all inhibited by the minimum MIC concentrations tested for at least two preservatives. High or low MIC values for one preservative, however, did not predict high MIC values for other preservatives, even when dealing with those of the same class. For example, RW138, which had the highest recorded MICs for MIT, BIT and PHE, was inhibited by a relatively low concentration of CITMIT (0.0009375%) and the lowest concentration of CHX tested (0.0005875%). In addition, the preservative susceptibility profiles were different for isolates of the same strain. For example strains sharing the AT-genotypes (section 5.2.2.) F460 (Pa#26 and RW110), 62CC (RW109 and RW192) and AFAA (RW130, RW131 and RW146) had varying responses to preservatives.

When relating the results to in-use concentrations of preservatives the CITMIT MIC values for 17 strains were above the EU cosmetics directive maximum permitted levels in personal care

products (0.0015%). The majority of these strains were from IND sources (n=11), fewer were of CF (n=2), CLIN (n=3) and ENV (n=1) origin. The CITMIT MIC for one IND strain, RW176 (MIC=0.005), was approximately 3 times higher than the permitted level. The PHE regulated level (1%) was only exceeded by the PHE MIC value for the IND strain RW138 (1.25%). Growth of the remaining strains was inhibited at levels below the EU maximum permitted concentrations.

The relationship between preservative susceptibility and isolation source was examined. The maximum preservative MIC values were observed for strains of IND origin across all of the preservatives (Figure 6.1), although in some cases, strains from other isolation sources shared these values; Pa#33 for CHX and Pa#19, Pa#26 and Pa#38 for BA. In addition, the higher isothiazolinone MIC values (≥0.00375% MIT, ≥ 0.001875% CITMIT and ≥ 0.02% BIT) were predominantly linked with IND strains. The lower isothiazolinone and PHE MIC values (≤ 0.000625% MIT, 0.0007815% CITMIT, 0.0015625% BIT and 0.234375% PHE) were predominantly linked with CF strains. Statistical analyses were performed to determine if the median preservative MICs of the groups (Table 6.3) from different isolation sources were significantly different from one another. The analyses included 39 strains; the 1 strain of unknown origin (RW198) was excluded. A Kruskal-Wallis test and post-hoc Wilcoxon tests with Benjamini Hochberg correction for non-parametric data were carried out for each preservative class using R statistical software. *P. aeruginosa* strains from IND sources were found to have significantly higher ($P < 0.05$) median preservative MICs than strains from CF sources for the preservatives MIT (0.0025 compared to 0.001875), CITMIT (0.0015626 compared to 0.0007815), BIT (0.01 compared to 0.005) and PHE (0.4688 compared to 0.2734). Furthermore, significant differences were found between the medians of CLIN and CF isolates, and ENV and CF isolates for PHE (0.4688 for CLIN and ENV isolates, compared to 0.2734 for CF isolates.

Table 6.1 Median and range MIC values of the isothiazolinone preservatives MIT, CITMIT and BIT

Strain	Other designations	Isolation source	MIC values (%)					
			MIT		CITMIT		BIT	
			Median	Range[a]	Median	Range	Median	Range
Pa#1	LESB58	CF	0.0000781	-	0.0002345	0.000156-0.000313	0.000313	-
Pa#4	C3719	CF	0.000313	-	0.0007815	0.00313-0,00125	0.000625	-
Pa#5	DK2	CF	0.001875	0.00125-0.0025	0.0009375	0.000625-0.000125	0.005	-
Pa#6	AES-1R	CF	0.0025	-	0.0007815	0.00313-0.00125	0.005	-
Pa#7	AUS23	CF	0.000313	-	0.0007815	0.00313-0.00125	0.00375	0.0025-0.005
Pa#8	AUS52	CF	0.000625	-	0.000313	-	0.0015625	0.000625-0.0025
Pa#17	PAO1	CLIN	0.001875	0.00125-0.005	0.0009375	0.000625-0.000125	0.0075	0.005-0.01
Pa#18	UCBPP-PA14	CLIN	0.00125	-	0.0009375	0.000625-0.000125	0.005	-
Pa#19	PAK	CLIN	0.00125	0.00125-0.0025	0.0007815	0.000625-0.000125	0.00375	0.0025-0.005
Pa#20	CHA	CF	0.0025	-	0.0009375	0.000625-0.000125	0.005	-
Pa#21	NN2	CF	0.001875	0.00125-0.005	0.0007815	0.000625-0.00025	0.005	-
Pa#26	39016	CLIN	0.0025	-	0.0009375	0.00313-0.00125	0.01	-
Pa#29	1709-12	CF	0.0025	0.0025-0.005	0.0007815	0.00313-0.00125	0.01	-
Pa#30	Mi 162	CLIN	0.00125	-	0.0007815	0.00313-0.00125	0.005	0.005-0.01
Pa#31	Jpn 1563	ENV	0.00375	0.0025-0.005	0.0015625	0.000625-0.00025	0.01	-
Pa#32	LMG 14084	ENV	0.0025	-	0.0009375	0.000625-0.00025	0.005	-
Pa#33	Pr335	ENV	0.001875	0.00125-0.0025	0.0009375	0.000625-0.00025	0.00375	0.0025-0.005
Pa#35	CPHL 9433	ENV	0.00375	0.0025-0.005	0.0009375	0.000625-0.00025	0.01	0.005-0.01
Pa#36	RP1	CF	0.00375	0.0025-0.005	0.0015625	0.000625-0.00025	0.005	-
Pa#38	57P31PA	CLIN	0.0025	-	0.0015625	0.000625-0.000125	0.01	-
Pa#40	39177	CLIN	0.001875	0.00125-0.0025	0.0009375	0.000625-0.000125	0.005	-
RW11	NCTC 12903; ATCC 27853	CLIN	0.00375	0.0025-0.005	0.0015625	0.000625-0.00025	0.01	0.005-0.01

RW109		IND	0.0025	0.0025-0.005	0.001875	0.00125-0.0025	0.01	-
RW110		IND	0.0025	0.0025-0.005	0.0015625	0.000625-0.00025	0.005	0.005-0.01
RW130		IND; DWL	0.00375	0.0025-0.005	0.0015625	0.000625-0.00025	0.02	-
RW131		IND; DWL	0.00375	0.0025-0.005	0.001875	0.00125-0.0025	0.02	-
RW138		IND; LAC	0.005	-	0.0009375	0.000625-0.000125	0.04	-
RW146		IND; DWL	0.005	-	0.0015625	0.000625-0.00025	0.02	-
RW149		IND; PC	0.00375	0.0025-0.005	0.0009375	0.000625-0.000125	0.01	-
RW151	ATCC 9027	CLIN	0.0025	-	0.0015625	0.000625-0.00025	0.01	-
RW172		IND; DWL	0.0025	-	0.001875	0.00125-0.0025	0.005	-
RW176		IND; DWL	0.005	-	0.005	-	0.02	-
RW184		IND; DWL	0.00125	0.00125-0.0025	0.0009375	0.000625-0.00025	0.005	0.0025-0.005
RW192		IND; SC	0.00375	0.0025-0.005	0.0015625	0.000625-0.00025	0.04	-
RW196	ATCC 13388; NCTC 8060	UNKNOWN	0.001875	0.00125-0.0025	0.00125	0.000625-0.000125	0.00375	0.0025-0.005
RW198	ATCC 15442	ENV	0.0025	0.0025-0.005	0.0015625	0.000625-0.00025	0.005	-
RW199		IND; MWF	0.00125	-	0.0009375	0.000625-0.000125	0.005	-
RW200		IND; TC	0.0025	0.00125-0.0025	0.0015625	0.000625-0.00025	0.005	-
RW202		IND; LAC	0.0025	-	0.0015625	0.000625-0.00025	0.01	-
RW204		IND; DWL	0.001875	0.00125-0.0025	0.0015625	0.000625-0.0025	0.01	0.005-0.01
Median MIC (%)			0.0025		0.0009375		0.005	
MIC range (%)			0.0000781-0.005		0.0002345-0.005		0.000313-0.04	
Maximum EU regulated levels (%)[b]			0.01		0.0015		0.2[c]	

Footnotes: MIC, minimum inhibitory concentration; MIT, methylisothiazolinone; CITMIT, chloromethylisothiazolinone and methylisothiazolinone blend in the ratio 3:1; BIT, benzisothiazolinone; CF, cystic fibrosis; CLIN, clinical; ENV, environmental; IND, industrial; DWL, dish wash liquid; LAC, liquid abrasive cleaner; PC, personal care; SC, Surface cleaner; MWF, metal working fluid; TC, timber care. Pa# strains were from the *P. aeruginosa* international reference panel collection held at Cardiff University, RW strains were from the RW collection held at Cardiff University. Median values are colour coded with darker shades reflecting an increase in MIC.
[a]The range is not reported where the replicate values were the same for a strain; [b] EU cosmetics directive 76/768/EEC, annex VI; [c]Not permitted in the EU, manufacturer's recommended level

Table 6.2 Median and range MIC values of the preservatives phenoxyethanol, chlorhexidine and benzoic acid

Strain	Other designations	Isolation source	MIC values (%)					
			PHE		CHX		BA	
			Median	Range	Median	Range	Median	Range
Pa#1	LESB58	CF	0.1171875	0.078125-0.15625	0.0046875	0.001563-0.00625	0.0125	
Pa#4	C3719	CF	0.234375	0.15625-0.3125	0.002344	0.000781-0.003125	0.0125	
Pa#5	DK2	CF	0.46875	0.3125-0.625	0.002344	0.001563-0.003125	0.05	
Pa#6	AES-1R	CF	0.234375	0.15625-0.3125	0.002344	0.000781-0.003125	0.05	
Pa#7	AUS23	CF	0.15625	0.15625-0.3125	0.001172	0.000781-0.001563	0.025	
Pa#8	AUS52	CF	0.234375	0.15625-0.3125	0.000781		0.05	
Pa#17	PAO1	CLIN	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	
Pa#18	UCBPP-PA14	CLIN	0.390625	0.015625-0.625	0.001953	0.000781-0.00625	0.05	
Pa#19	PAK	CLIN	0.46875	0.3125-0.625	0.002344	0.001563-0.003125	0.05	
Pa#20	CHA	CF	0.46875	0.3125-0.625	0.003125	0.001563-0.003125	0.05	
Pa#21	NN2	CF	0.3125	0.15625-0.3125	0.0046875	0.003125-0.00625	0.05	
Pa#26	39016	CLIN	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	
Pa#29	1709-12	CF	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	
Pa#30	Mi 162	CLIN	0.46875	0.3125-0.625	0.002344	0.001563-0.003125	0.05	
Pa#31	Jpn 1563	ENV	0.46875	0.3125-0.625	0.0046875	0.001563-0.00625	0.075	0.1 - 0.05
Pa#32	LMG 14084	ENV	0.625	0.3125-0.625	0.0039065	0.001563-0.00625	0.05	
Pa#33	Pr335	ENV	0.625	0.3125-0.625	0.00625		0.05	
Pa#35	CPHL 9433	ENV	0.46875	0.3125-0.625	0.003125		0.05	
Pa#36	RP1	CF	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	
Pa#38	57P31PA	CLIN	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	
Pa#40	39177	CLIN	0.625	0.3125-0.625	0.0046875	0.003125-0.0125	0.05	

RW11	NCTC 12903; ATCC 27853	CLIN	0.46875	0.3125-0.625	0.002344	0.001563-0.00625	0.075	0.1-0.05
RW109		IND	0.46875	0.3125-0.625	0.0046875	0.001563-0.00625	0.05	
RW110		IND	0.46875	0.3125-0.625	0.00625		0.05	0.1-0.05
RW130		IND; DWL	0.625	-	0.002344	0.001563-0.003125	0.05	0.05
RW131		IND; DWL	0.46875	0.3125-0.625	0.002344	0.001563-0.003125	0.05	0.05
RW138		IND; LAC	1.25	1.25-2.5	0.00058575	0.000391- 0.001563	0.05	0.05
RW146		IND; DWL	0.46875	0.3125-0.625	0.002344	0.001563-0.003125	0.05	0.1-0.05
RW149		IND; PC	0.625	0.625	0.002344	0.001563-0.003125	0.05	0.05
RW151	ATCC 9027	CLIN	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	0.05
RW172		IND; DWL	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	0.05
RW176		IND; DWL	0.46875	0.3125-0.625	0.002344	0.001563-0.003125	0.05	0.05
RW184		IND; DWL	0.234375	0.15625-0.625	0.000781		0.05	0.05
RW192		IND; SC	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	0.05
RW196	ATCC 13388; NCTC 8060	UNKNOWN	0.46875	0.3125-0.625	0.001172	0.000781-0.001563	0.05	0.05
RW198	ATCC 15442	ENV	0.46875	0.3125-0.625	0.00625	0.003125-0.0125	0.05	0.05
RW199		IND; MWF	0.625	0.625	0.001172	0.000781-0.003125	0.05	0.05
RW200		IND; TC	0.46875	0.3125-0.625	0.0046875	0.003125-0.00625	0.05	0.05
RW202		IND; LAC	0.46875	0.3125-0.625	0.00625		0.05	0.05
RW204		IND; DWL	0.46875	0.3125-0.625	0.0046875	0.003125-0.0125	0.05	0.05
Median MIC (%)			0.46875		0.00351575		0.05	
MIC range (%)			0.1171875-1.25		0.00058575-0.00625		0.0125-0.075	
Maximum EU regulated levels (%)[b]			1		0.3		0.5	

Footnotes: MIC, minimum inhibitory concentration; PHE, phenoxyethanol; CHX, chlorhexidine; BA, benzoic acid at pH5; CF, cystic fibrosis; CLIN, clinical; ENV, environmental; IND, industrial; DWL, dish wash liquid; LAC, liquid abrasive cleaner; PC, personal care; SC, Surface cleaner; MWF, metal working fluid; TC, timber care. Pa# strains were from the *P. aeruginosa* international reference panel collection held at Cardiff University, RW strains were from the RW collection held at Cardiff University. Median values are colour coded with darker shades reflecting an increase in MIC.
[a]The range is not reported where the replicate values were the same for a strain; [b] EU cosmetics directive 76/768/EEC, annex VI

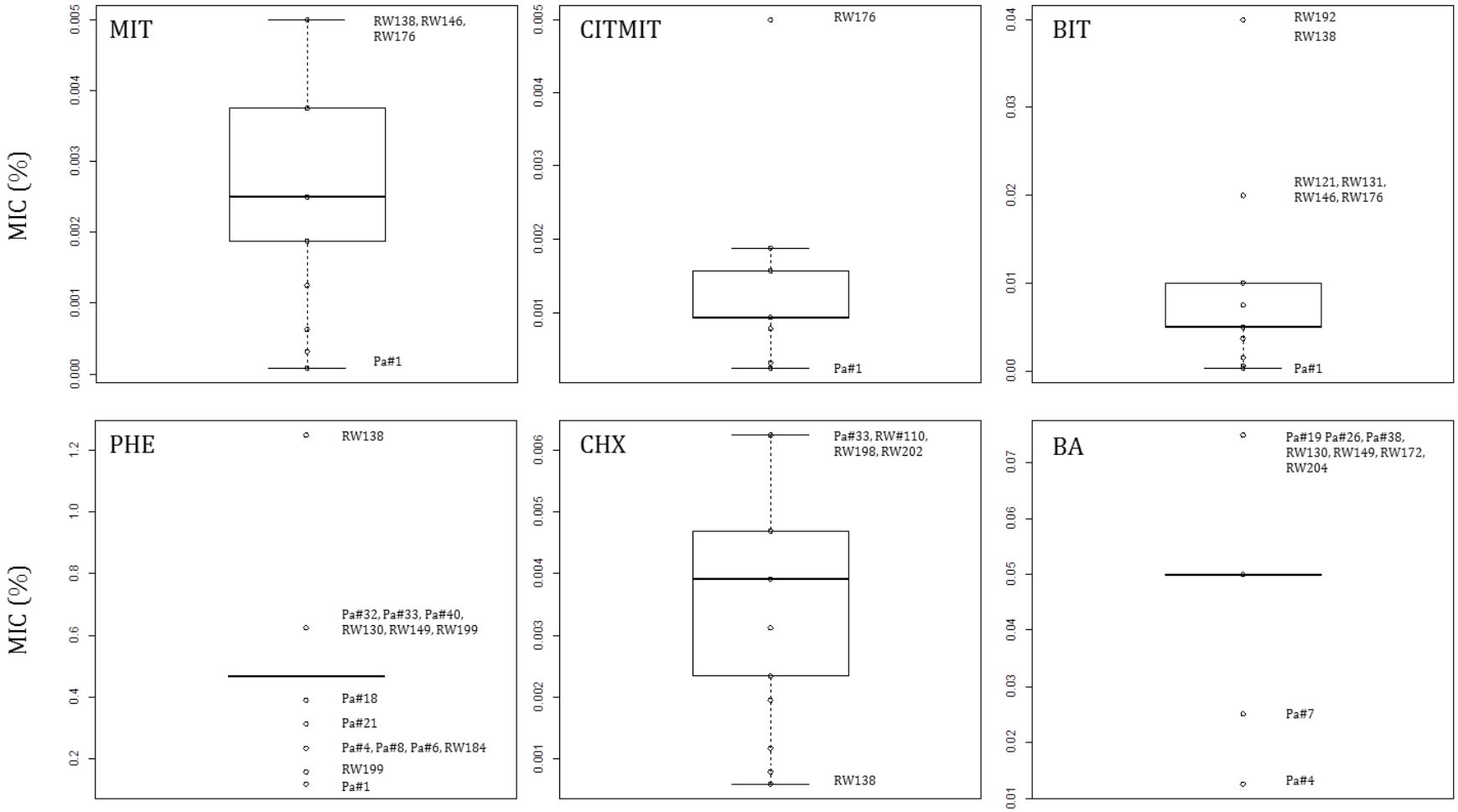


Figure 6.1 Minimum inhibitory concentrations (MICs) of preservatives for a panel of 40 *P. aeruginosa* strains. The distribution of the MIC data was visualised using boxplots which display the median, upper quartile, lower quartile, maximum and minimum values. Data points are shown by open circles, with outliers indicated outside of the boxplots. The strain names of outliers and those with the minimum and maximum MIC values are given to the right of the data points. In cases where more than one strain had the same MIC, all of the strains are listed. MIT, methylisothiazolinone; CITMIT, chloromethylisothiazolinone and methylisothiazolinone blend in the ratio 3:1; BIT, benzisothiazolinone; PHE, phenoxyethanol; CHX, chlorhexidine; BA, benzoic acid.

Table 6.3 Median preservative MICs of preservatives for strains from different isolation sources

Isolation source	Median preservative MIC (%)					
	MIT	CITMIT	BIT	PHE	CHX	BA
CF	0.001875	0.0007815	0.005	0.2734	0.002734	0.05
CLIN	0.001875	0.0009375	0.0075	0.4688	0.004687	0.05
ENV	0.0025	0.0009375	0.005	0.4688	0.004687	0.05
IND	0.0025	0.0015625	0.01	0.4688	0.002344	0.05

Footnotes:
MIC, minimum inhibitory concentration; PHE, phenoxyethanol; CHX, chlorhexidine; BA, benzoic acid at pH 5; CF, cystic fibrosis; CLIN, clinical; ENV, environmental; IND, industrial

6.2.1.2 Preservative combination testing

The efficacy of an isothiazolinone combination was investigated using two *P. aeruginosa* strains from IND sources; RW109, a strain used in preservative efficacy testing, and RW176, a strain that possessed the highest MIC value for both MIT and BIT (section 6.2.1.1). The relationship between the preservatives MIT and BIT was determined using checkerboard testing and interpreted using the FIC index (sections 2.12.2.1 and 2.12.2.2), the results of which are shown in Table 6.4. For both RW109 and RW138 the ΣFIC was 1 suggesting a relationship where neither preservative agent influenced the MIC when in combination.

6.2.1.2 Evaluation of preservative enhancing agents

The antimicrobial activity of combinations of preservatives and preservative enhancing agents was investigated using checkerboard testing, and interpreted using the FIC index. The combinations investigated were: (1) the isothiazolinone preservative CITMIT and the efflux pump inhibitor phenyl-arginine-beta-naphthylamide (PAβN), and; (2) the preservative PHE against the preservative enhancers CX3, CX7 and Velsan SC (VSC). The CITMIT and PAβN combination was evaluated using 10 *P. aeruginosa* strains (Pa#17, Pa#18, RW11, RW109, RW110, RW131, RW176, RW192, RW196, and RW198) including the strain with the highest MIC value for CITMIT (RW176). The PHE and preservative enhancer combinations were evaluated using two strains, the preservative efficacy testing strain RW109 and the strain with the highest PHE MIC value, RW138. The relationships between CITMIT and PAβN and PHE and CX7 are shown in Table 6.5 and Table 6.6, respectively. The combination of PHE and PAβN yielded ΣFIC results of between 0.5 and 1 for 6 strains (RW109, Pa#17, Pa#18, RW110, RW192 and RW198) suggesting an additive relationship between the two agents in these cases. For 3 other strains (RW176, RW11 and RW131) the combination of PHE and PAβN was more efficacious, yielding ΣFIC values of less than 0.5, indicating a synergistic relationship. For the strain RW196 the PHE and PAβN combination was less efficacious and had a ΣFIC of 2. The activity of the PHE and CX7 combination was additive, with both strains having a ΣFIC of 0.54. The relationships between PHE and the two other preservative enhancers, CX3 and VSC, were likely to be antagonistic as follows; the addition of CX3 (0 to 2.5%) doubled the MIC of PHE (0.4 to 0.8%), and the addition of VSC (0 to 4%) increased the MIC of PHE to above the range tested (>1%).

Table 6.4 Activity of an isothiazolinone combination against *P. aeruginosa*

Strain	Preservative agents in combination				ΣFIC	Relationship
	MIT (%)		BIT (%)			
	MIC (MIT+BIT)/ MIC (MIT)	FIC	MIC (BIT+MIT)/ MIC (BIT)	FIC		
RW109	0.005/0.01	0.5	0.005/0.01	0.5	1	Indifferent
RW138	0.0025/0.005	0.5	0.005/0.01	0.5	1	Indifferent

Footnotes: MIT, methylisothiazolinone; BIT, benzisothiazolinone; MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration; ΣFIC, fractional inhibitory concentration index.

Table 6.5 Activity of CITMIT and PAβN combinations against *P. aeruginosa*

Strain	Preservative agents in combination				ΣFIC	Relationship
	CITMIT (%)		PABN (mg/ml)			
	MIC (CITMIT + PAβN)/ MIC (PAβN)	FIC	MIC (PAβN + CITMIT)/ MIC (PAβN)	FIC		
RW109	0.0005/0.001	0.5	50/250	0.2	0.7	Additive
RW176	0.0005/0.0003	0.167	50/250	0.2	0.367	Synergistic
Pa#17	0.0002/0.0003	0.667	25/500	0.05	0.717	Additive
Pa#18	0.0002/0.0004	0.5	25/500	0.05	0.55	Additive
RW11	0.0002/0.0005	0.4	25/500	0.05	0.45	Synergistic
RW110	0.0002/0.0004	0.5	25/500	0.05	0.55	Additive
RW131	0.0001/0.0004	0.25	50/500	0.1	0.35	Synergistic
RW192	0.0002/0.0004	0.5	50/500	0.1	0.6	Additive
RW196	0.0004/0.0004	1	25/25	1	2	Indifferent
RW198	0.0002/0.0005	0.4	50/500	0.1	0.5	Additive

Footnotes: CITMIT, chloromethylisothiazolinone and methylisothiazolinone blend in the ratio 3:1; PAβN, L-Phe-Arg-β-napthylamide; MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration; ΣFIC, fractional inhibitory concentration index.

Table 6.6 Activity of the PHE and CX7 combination against *P. aeruginosa*

Strain	Preservative agents in combination				ΣFIC	Relationship
	PHE (%)		CX7 (%)			
	MIC (PHE+CX7)/ MIC (PHE)	FIC	MIC (CX7+PHE)/ MIC (CX7)	FIC		
RW109	0.2/0.4	0.5	0.1/2.5	0.04	0.54	Additive
RW138	0.2/0.4	0.5	0.1/2.5	0.04	0.54	Additive

Footnotes: PHE, phenoxyethanol, CX7, CX7 preservative enhancer; MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration; ΣFIC, fractional inhibitory concentration index.

6.2.2 Phenotypic characterisation

6.2.2.1 Growth curve analysis

The growth dynamics of the panel strains in MH broth at 37°C over 24 hours were determined using a Bioscreen C microbial growth analyser. The growth curves (Figure 6.2) illustrated considerable variability in growth dynamics between strains. Under these conditions the majority of the *P. aeruginosa* strains reached their maximum culture density by 15 hours and split largely into two groups; those reaching a higher culture density (>0.20) and those reaching a lower culture density (<0.20). This has been illustrated in greater detail in the boxplot in Figure 6.3, which resolved the maximum culture density data for the 40 panel strains into two broad groups. From this boxplot it appears that the majority of IND strains (13/15), CLIN strains (7/9), and all ENV strains (5/5) reached an OD greater than 0.20 after 15 hours. The OD values CF strains were distributed fairly evenly throughout the boxplot in Figure 6.3.

To more comprehensively model the growth curve parameters (length of lag phase, maximum growth rate and maximum culture density reached) the grofit package for R statistical software was used, with data from up to 24 hours growth. The *P. aeruginosa* strains Pa#1, RW130, RW131, RW138, RW146, RW149 and RW196 were excluded from the analysis as they produced growth curves that could not be modelled accurately by grofit, possibly because of their long lag phase and/or poorly defined exponential phase. Growth parameters for the remaining 33 strains are displayed in Table 6.7 and the spread of data summarised by boxplots (Figure 6.4). Outliers in terms of growth kinetics were as follows (Figure 6.4): strain RW172 had a longer lag phase (8.1 hours) than the maximum value for all strains (upper whisker = 7.1 hours); strain RW184 had a lower growth rate (0.02 $h^{-1)}$ than the minimum value for IND strains (lower whisker = 0.06); strains Pa#4, Pa#7 and Pa#8 reached a lower culture density (log_{10} Max OD values 0.19, 0.19 and 0.16 respectively) than the minimum value for all strains (lower whisker = 0.24), and RW184 had a lower culture density (log_{10} Max OD = 0.24) than the minimum value for IND strains (lower whisker = 0.31). To examine the relationship between growth dynamics and isolation source statistical analyses were performed; a Kruskal-Wallis test and post-hoc Wilcoxon tests with Benjamini-Hochberg correction for non-parametric data were carried out for each growth parameter class using R statistical software. There were no significant differences at the p=0.05 level between any of the group medians for lag phase, growth rate or maximal OD.

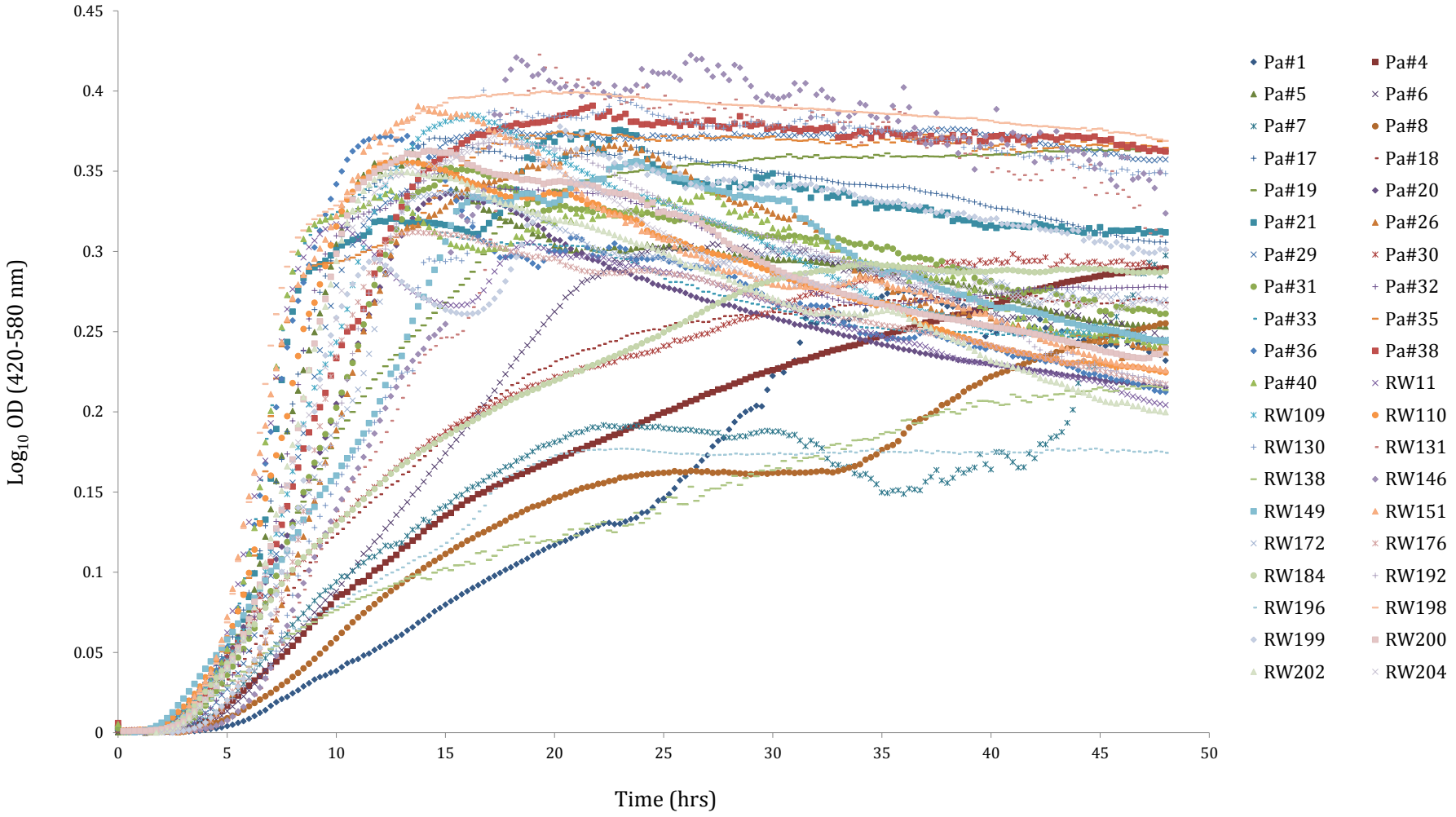


Figure 6.2 Growth curves of the 40 *P. aeruginosa* panel strains. The growth of the strains in MH broth at 37°C was monitored by culture optical density over 24 hours.

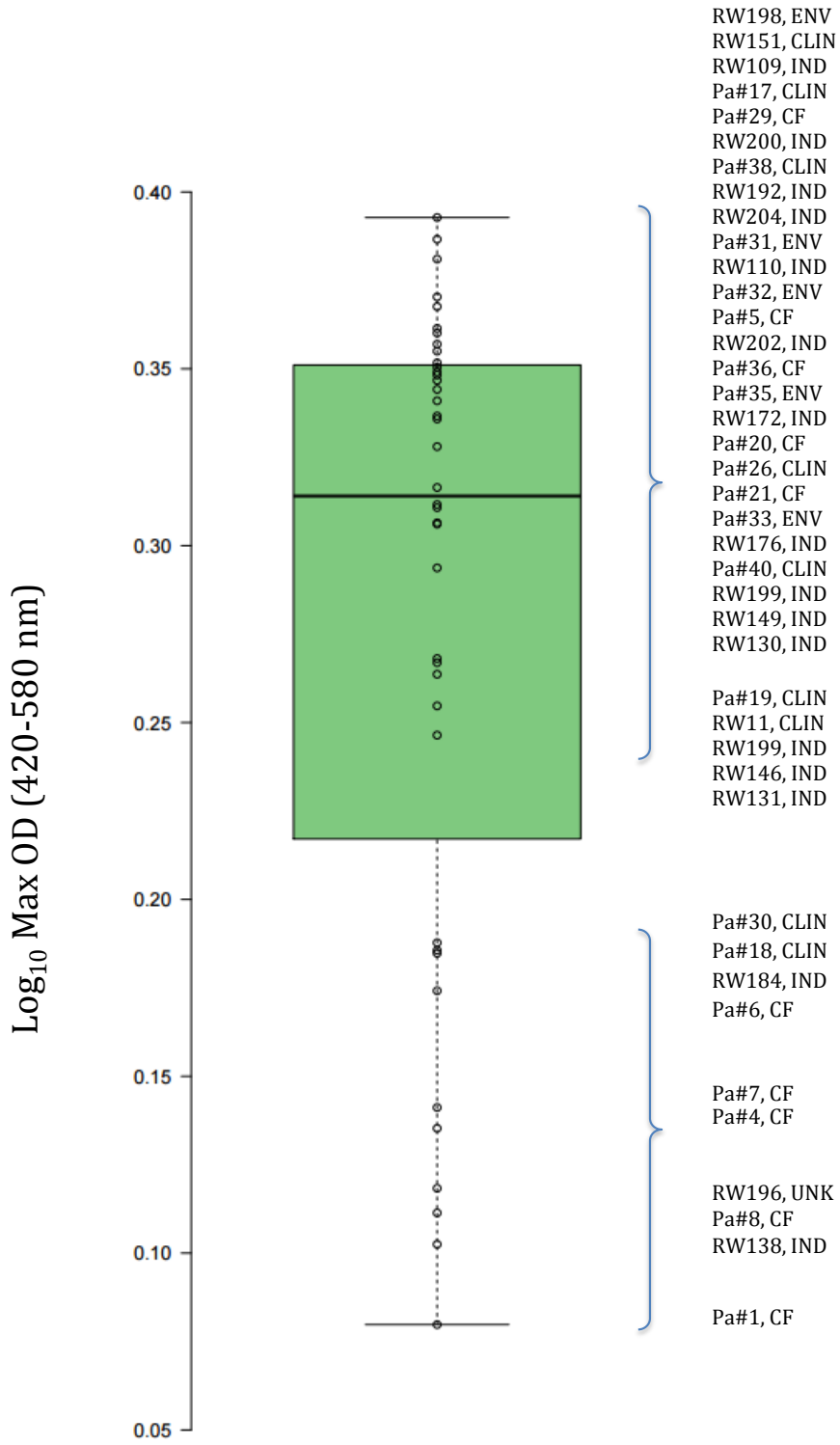


Figure 6.3 Maximum culture density reached at 15 hours growth by the 40 *P. aeruginosa* panel strains. The distribution of the maximum culture density data at 15 hours growth was visualised using a boxplot displaying the median, upper quartile, lower quartile, maximum and minimum values. Data points for each of the strains are included on the plot. The blue brackets illustrate three broad strain groupings (high, intermediate and low culture density) and the strain names are listed in order (highest to lowest Max OD) to the right of the boxplot, along with isolation source (CF, cystic fibrosis; CLIN, clinical; ENV environmental; IND, industrial; UNK, unknown).

Table 6.7 Growth parameters of 33 *P. aeruginosa* strains after 24 hours growth

Strain	Other designations	Source	Lag phase (hrs)	Growth rate (hrs^{-1})	Log_{10} Max OD (420-580)
Pa#4	C3719	CF	4.45	0.02	0.19
Pa#5	DK2	CF	4.99	0.07	0.36
Pa#6	AES-1R	CF	6.65	0.02	0.30
Pa#7	AUS23	CF	3.92	0.02	0.19
Pa#8	AUS52	CF	5.51	0.01	0.16
Pa#17	PAO1	CLIN	5.14	0.07	0.37
Pa#18	UCBPP-PA14	CLIN	3.62	0.02	0.25
Pa#19	PAK	CLIN	3.69	0.03	0.35
Pa#20	CHA	CF	4.84	0.04	0.34
Pa#21	NN2	CF	5.68	0.12	0.38
Pa#26	39016	CLIN	6.79	0.06	0.37
Pa#29	1709-12	CF	5.87	0.07	0.38
Pa#30	Mil 162	CLIN	2.61	0.02	0.24
Pa#31	Jpn 1563	ENV	6.20	0.05	0.35
Pa#32	LMG 14084	ENV	5.76	0.09	0.35
Pa#33	Pr335	ENV	4.88	0.10	0.34
Pa#35	CPHL 9433	ENV	5.53	0.13	0.37
Pa#36	RP1	CF	4.67	0.08	0.37
Pa#38	57P31PA	CLIN	4.83	0.05	0.39
Pa#40	39177	CLIN	4.98	0.10	0.34
RW11	NCTC 12903; ATCC 27853	CLIN	5.18	0.11	0.32
RW109		IND	5.24	0.06	0.38
RW110		IND	5.21	0.08	0.36
RW151	ATCC 9027	CLIN	4.11	0.08	0.39
RW172		IND; DWL	8.06	0.07	0.34
RW176		IND; DWL	6.66	0.08	0.31
RW184		IND; DWL	2.95	0.02	0.24
RW192		IND; SC	7.12	0.07	0.37
RW198	ATCC 15442	ENV	4.94	0.12	0.40
RW199		IND; MWF	6.65	0.08	0.37
RW200		IND; TC	5.74	0.08	0.36
RW202		IND; DWL	5.33	0.07	0.35
RW204		IND; DWL	6.89	0.08	0.37

Footnotes: CF, cystic fibrosis; CLIN, clinical, ENV, environmental; IND, industrial; DWL, dish wash liquid; LAC, liquid abrasive cleaner; SC, surface cleaner; MWF, metal working fluid; TC, timber care

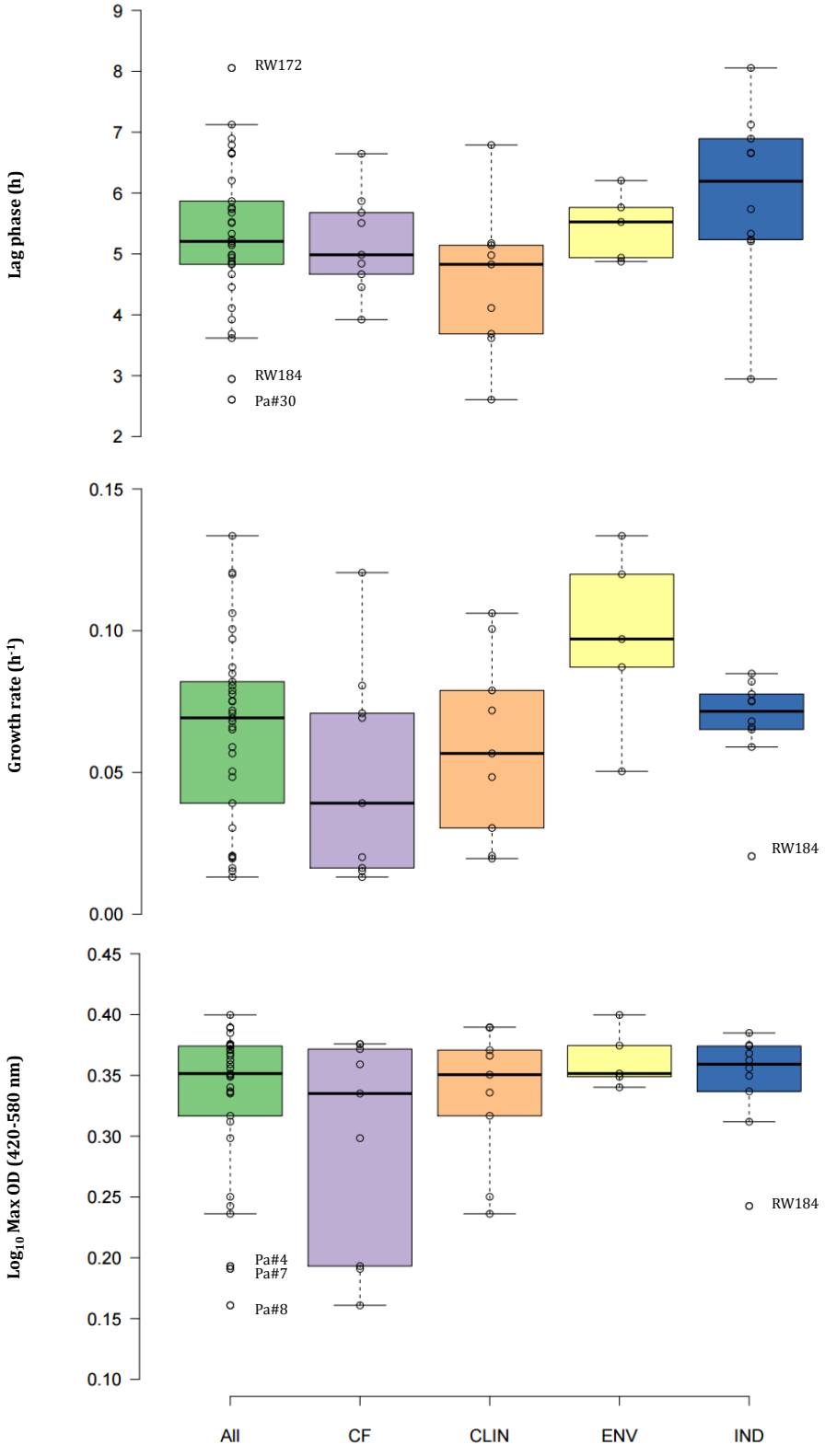


Figure 6.4 Growth parameters of 33 *P. aeruginosa* strains at 24 hours growth. The distribution of the data (length of lag phase, growth rate and maximum culture density reached) was visualised using boxplots which display the median, upper quartile, lower quartile, maximum and minimum values. Outliers are indicated by open circles. Strains included in the analysis have either been grouped together (All strains) or by isolation source (CF, cystic fibrosis; CLIN, clinical; ENV environmental; IND, industrial). Strain names have been included next to outliers.

6.2.2.2 6.2.2.2 Motility assays

The 40 *P. aeruginosa* panel strains were assayed for swimming, swarming and twitching motilities. The diameters of the zones of motility are shown in Table 6.8, and representative photographs given in Figure 6.5. Motility was variable across the panel with the majority of strains (n=37) displaying at least one form. The three strains that were non-motile were of CF (n=2) and IND (n=1) origin. Across the rest of the panel, one did not display swarming motility (Pa#7), seven were deficient in swarming on at least one type of agar and twitching (Pa#1, Pa#6, Pa#29, Pa#30, Pa#32, RW196 and RW198), and four did not display twitching motility (RW121, RW138, RW149 and RW151). These strains were from CF, CLIN, ENV and IND sources; there did not appear to be a link between isolation source and motility. Only one strain (RW200) demonstrated 'true' swarming, indicated by the formation of finger-like projections radiating from the inoculation point, observed only on the minimal medium BSM-G 0.5% agar (Figure 6.5). Three strains of IND origin (RW130, RW131 and RW146), determined in the previous chapter to have the same AT-genotype (AFAA) and isolated from DWL products in Italy, displayed similar unusual swimming motilities. Rather than a uniform swim zone radiating from the inoculation point the, swimming pattern consisted of a central zone with an uneven growth boundary (Figure 6.6).

Table 6.8 Swimming, swarming and twitching motilities of the *P. aeruginosa* panel

Strain	Other designations	Source	Swimming diameter (mm)	Swarming diameter (mm)		Twitching diameter (mm)
				LB 0.5% agar	BSM-G 0.5% agar	
Pa#1	LESB58	CF	17.5	-	-	-
Pa#4	C3719	CF	-	-	-	-
Pa#5	DK2	CF	53.5	19	12.5	12
Pa#6	AES-1R	CF	51	-	-	-
Pa#7	AUS23	CF	52.5	-	-	9
Pa#8	AUS52	CF	-	-	-	-
Pa#17	PAO1	CLIN	60.5	28	18.5	23.5
Pa#18	UCBPP-PA14	CLIN	62.5	24	16.5	11
Pa#19	PAK	CLIN	60	12.5	10	17
Pa#20	CHA	CF	80	25.5	18	19.5
Pa#21	NN2	CF	61	12	10	20.5
Pa#26	39016	CLIN	75	22.5	11	30.5
Pa#29	1709-12	CF	29.5	7.5	-	-
Pa#30	Mi 162	CLIN	62.5	-	-	-
Pa#31	Jpn 1563	ENV	25	16.5	15	38
Pa#32	LMG 14084	ENV	58.5	14.5	-	-
Pa#33	Pr335	ENV	53.5	16	11.5	18.5
Pa#35	CPHL 9433	ENV	66	13.5	7.5	11.5
Pa#36	RP1	CF	65	26	8.5	15.5
Pa#38	57P31PA	CLIN	68.5	31	14	14
Pa#40	39177	CLIN	48.5	15.5	8	19.5
RW11	NCTC 12903; ATCC 27853	CLIN	79	15.5	10	16
RW109		IND	55	17	14	8
RW110		IND	80	22	14.5	31.5
RW130		IND; DWL	27.5[a]	8	7	-
RW131		IND; DWL	27.6[a]	8.5	7.5	-
RW138		IND; LAC	23.5	11.5	12.5	8.75
RW146		IND; DWL	25.5[a]	9.5	9	-
RW149		IND; PC	61.5	8	7	-
RW151	ATCC 9027	CLIN	71.5	31	22.5	15.5
RW172		IND; DWL	58	16	8	18
RW176		IND; DWL	63.5	40	15	24.5
RW184		IND; DWL	-	-	-	-
RW192		IND; SC	32.5	18	9.5	16.5
RW196	ATCC 13388; NCTC 8060	UNKNOWN	64	9.5	-	-
RW198	ATCC 15442	ENV	71.5	8.5	-	-
RW199		IND; MWF	35.5	8.5	8	13
RW200		IND; TC	75	24	60[b]	39.5
RW202		IND; LAC	76	25	16	36
RW204		IND; DWL	76.5	32	35	38

Footnotes: CF, cystic fibrosis; CLIN, clinical; ENV, environmental; IND, industrial; DWL, dish wash liquid; LAC, liquid abrasive cleaner; PC, personal care; SC, Surface cleaner; MWF, metal working fluid; TC, timber care, '-' designates non motile, and light blue shading indicates highly motile.
[a]Strains exhibited an atypical swimming motility shown in Figure 6.6
[b]Strain exhibited typical swarming with a diameter of approximately 60 mm shown in Figure 6.5

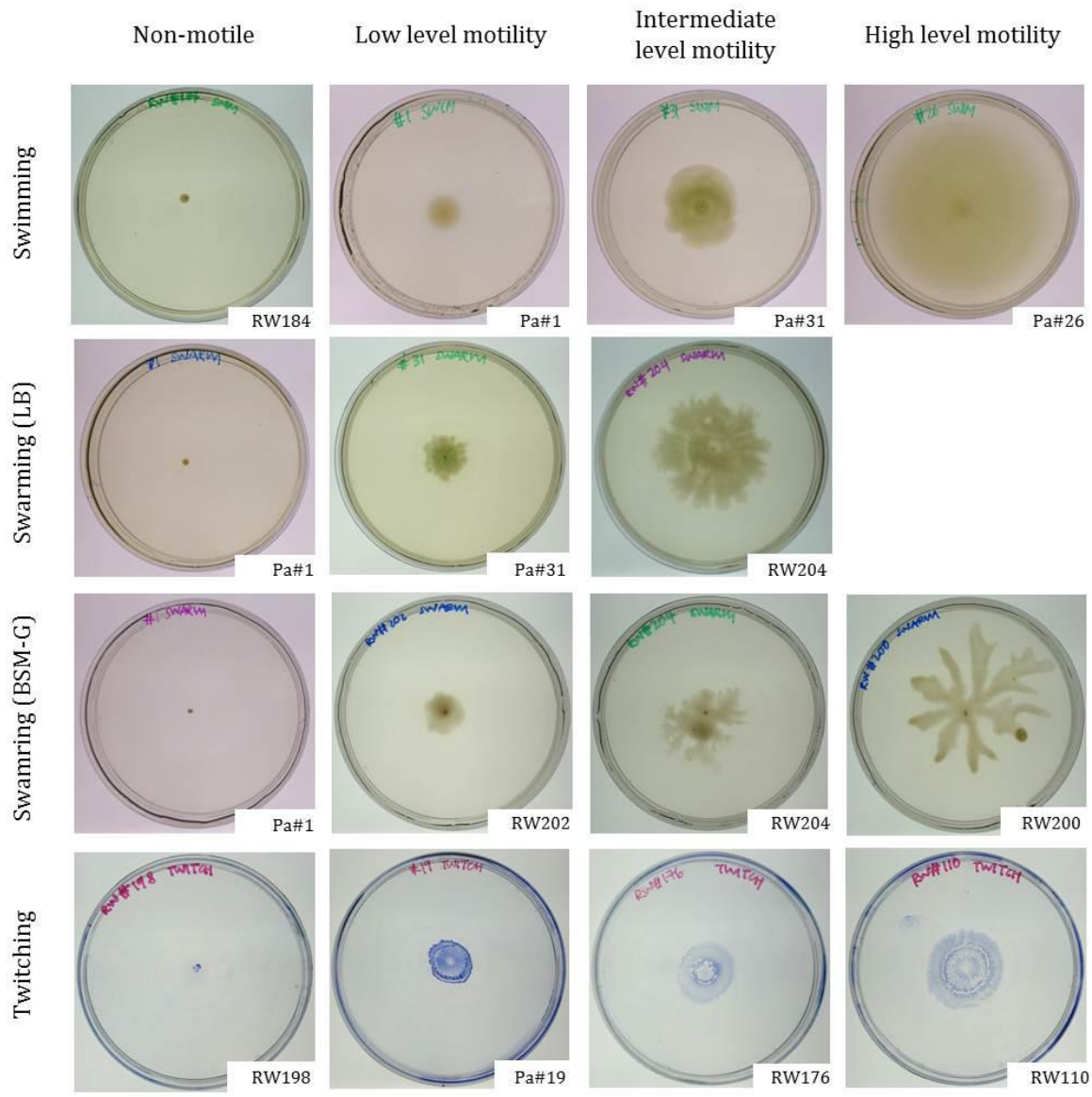


Figure 6.5 Swimming, swarming and twitching motilities of the *P. aeruginosa* panel strains. Representative images of the levels of swimming (0.3% LB agar), swarming (0.5% LB and BSM-G agar) and twitching (1% LB agar) motility exhibited by panel strains. Results were recorded after 16-18 hours incubation at 30°C (swarming) or 37°C (swimming and twitching) High level swarming motility was not observed for any panel strains on LB.

Figure 6.6 Atypical swimming motilities of the industrial strains RW130, 131 and 146. Results were recorded after 16-18h growth on 0.3% LB agar at 37°C.

6.2.3 Evolution of preservative resistance using planktonic growth and biofilm models

P. aeruginosa is a highly adaptable bacterial species as shown by the diversity of environments it can be isolated from and its phenotypic variability. The absence of genetic lineages associated with industrial contamination (Chapter 5), and the fact that the highest MIC values observed for the preservatives MIT, CITMIT, BIT and PHE were for IND strains, suggested that *P. aeruginosa* is a diverse species able to adapt to preservatives. The development of adaptive resistance to preservatives by *P. aeruginosa* was examined using planktonic (Wong *et al.* 2012), and biofilm (Poltak and Cooper 2011) growth models. The experiments were performed with the strain PA14 as a well-studied *P. aeruginosa* reference isolate and one of the most widely distributed *P. aeruginosa* clone types (Tümmler *et al.* 2014).

6.2.3.1 Susceptibility of *P. aeruginosa* PA14 to a preservative combination

The preservative susceptibility of *P. aeruginosa* PA14 to the preservative combination CITMIT-DMDMH-EDTA (100% strength constituted 0.0009% CITMIT, 0.22% DMDMH and 0.1% EDTA) was examined to establish sub-inhibitory starting concentrations for both the planktonic and biofilm growth experiments. Using the Bioscreen C instrument to monitor the growth dynamics of *P. aeruginosa* PA14 for 48 hours at 37°C it was determined that growth of the strain was inhibited by concentrations of ≥10% of CITMIT-DMDMH-EDTA (Figure 6.7). A lower concentration of 7.5% CITMIT-DMDMH-EDTA inhibited growth until approximately 35 hours, indicating that this concentration greatly extended lag phase. At 5% CITMIT-DMDMH-EDTA the growth curve resembled that of the TSB growth control, but had a lag phase of approximately 5

hours longer (Figure 6.7). From these results a concentration of 2.5% CITMIT-DMDMH-EDTA was selected to start the planktonic growth experiment where transfers were made every 24 hours. A concentration of 7.5% CITMIT-DMDMH-EDTA was selected to start the biofilm growth experiment as transfers were made every 48 hours, allowing more time for growth in the preservative combination.

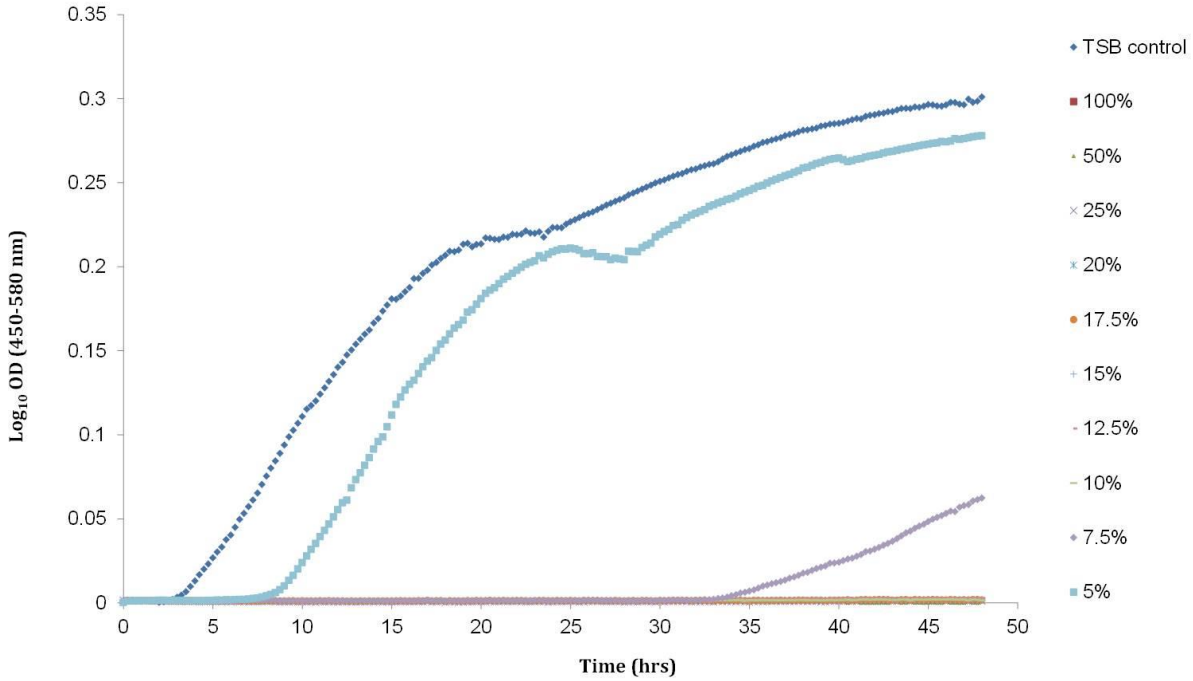


Figure 6.7 Preservative susceptibility of *P. aeruginosa* PA14 to the preservative combination CITMIT-DMDMH-EDTA. The growth of PA14 at 37°C in TSB and different concentrations of the preservative combination CITMIT-DMDMH-EDTA (5-100%) was monitored by culture optical density over 48 hours. The 100% strength preservative combination constituted 0.0009% CITMIT, 0.22% DMDMH and 0.1% EDTA.

6.2.3.2 Adaptation of planktonic cultures to a preservative combination

Using a selection experiment similar to that of Wong *et al.* (Wong *et al.* 2012)(section 2.15.2) planktonic populations of *P. aeruginosa* PA14 were evolved under three different conditions; (1) TSB; (2) TSB with 2.5% preservative combination, and; (3) TSB with increasing amounts of the preservative combination CITMIT-DMDMH-EDTA. Every 24 hours for 8 days, populations were propagated by being transferred into fresh growth medium, with ten replicate cultures for each condition (see section 2.15.2 for full details). Growth was observed after every transfer for all of the replicates grown in TSB and TSB-2.5% preservative combination conditions. For the TSB-increasing preservative concentration condition, not all replicates adapted at the same rate

yielding populations with varying tolerances; the highest concentrations that the replicates grew in were 10% (1 replicate), 12.5% (8 replicates), and 15% (1 replicate). A selection of end-point isolates were chosen for further preservative susceptibility testing (section 6.2.3.4), to determine stability of phenotype.

6.2.3.3 Adaptation of biofilm growth to a preservative combination

A month-long experiment examined the adaptation of *P. aeruginosa* PA14 biofilms to a preservative combination, using a model adapted from Poltack and Cooper (Poltak and Cooper 2011)(Section 2.15.3). Biofilm populations grown on plastic beads were transferred into either fresh TSB or TSB with increasing increments of the preservative combination every 48 hours. The starting concentration was 7.5% CITMIT-DMDMH-EDTA and the end concentration was 20% CITMIT-DMDMH-EDTA. A planktonic growth control in TSB was also performed alongside the biofilm model. The starting inoculum for the experiment was approximately 10^5 cfu/ml and approximately 10^7 cfu/ml were recovered from the end sample planktonic growth from the TSB control and TSB of the biofilm growth populations. The number of viable cells recovered from the end sample bead-grown biofilms (TSB and TSB plus preservative combination conditions) was also approximately 10^7. Due to problems encountered in obtaining viable counts from samples taken during the experiment, no extra viable count data was recorded.

Six days after the start of the experiment a small colony variant was observed in all populations, in addition to the original spreading colonies of the wild-type *P. aeruginosa* PA14. The small colony variant occurred in high numbers in all conditions, frequently equivalent to or greater than the ancestral PA14 morphology, although exact proportions were not recorded at this sample point. Furthermore, one more colony morphotype was observed in the TSB biofilm population; a spreading colony with a ruffled surface (Figure 6.8). This ruffled spreading colony type was observed at low frequency and not seen in the TSB plus CITMIT-DMDMH-EDTA biofilm populations. At the end of the experiment a mixture of different colony morphologies were observed suggesting adaptation to the different conditions, but there was no specific adaptation of a single morphotype to one condition (Table 6.9). A selection of end-point isolates were chosen for preservative susceptibility testing (section 6.2.3.4). To obtain populations representative of the three experimental conditions the two biological replicates were pooled for end-point samples of planktonic growth, biofilm growth from the beads of the TSB growth conditions and biofilm growth from the beads of the TSB plus CITMIT-DMDMH-EDTA growth condition. These pooled samples were also included the preservative susceptibility testing.

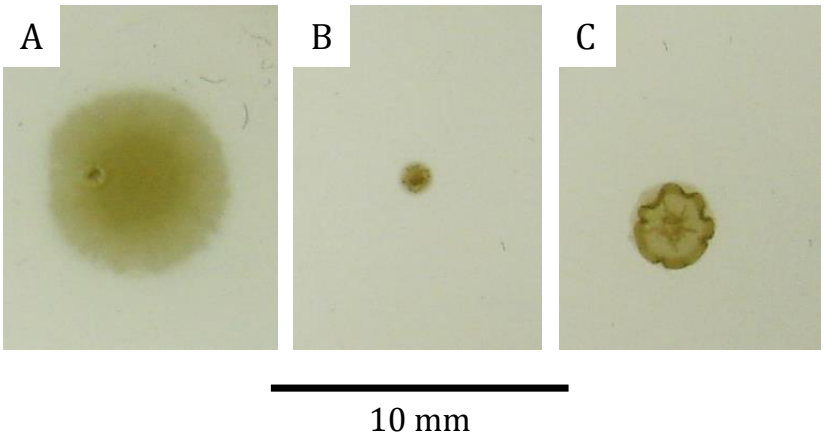


Figure 6.8 *P. aeruginosa* PA14 colony morphotypes observed during the biofilm adaptation experiment. Representative images of the three different colony morphotypes that emerged after day six of the experiment are shown: (A) the spreading colony morphology of the ancestral *P. aeruginosa* PA14, seen for all three conditions; (B) a small colony variant, also observed for all three conditions, and; (C) a spreading ruffled colony type, seen in only the TSB biofilm population. The black scale bar represents 10 mm.

Table 6.9 *P. aeruginosa* PA14 colony morphotypes isolated at the end of the biofilm experiment

Experimental conditions	Description	Total viable count
TSB planktonic growth replicate 1	Small	2.2×10^7 cfu/ml
	Spreading	2×10^5 cfu/ml
TSB planktonic growth replicate 2	Small	1.6×10^7 cfu/ml
	Spreading	8×10^5 cfu/ml
TSB Bead replicate 1	Small	1.8×10^7
	Spreading ruffled	1×10^5
TSB Bead replicate 2	Small ruffled	3.5×10^7
TSB Bead planktonic growth replicate 1	Small	4.1×10^7 cfu/ml
	Spreading	2×10^5 cfu/ml
	Spreading ruffled	1×10^5 cfu/ml
TSB Bead planktonic growth replicate 2	Small ruffled	3.6×10^7 cfu/ml
TSB 20 % CITMIT-DMDMH-EDTA Bead replicate 1	Small	2.5×10^7
	Small ruffled	
TSB 20 % CITMIT-DMDMH-EDTA Bead replicate 2	Small	3×10^6
	Spreading	1.4×10^7
TSB 20 % CITMIT-DMDMH-EDTA Bead planktonic growth replicate 1	Small	3.5×10^7 cfu/ml
	Small ruffled	
TSB 20 % CITMIT-DMDMH-EDTA Bead planktonic growth replicate 2	Small	2×10^5 cfu/ml
	Spreading	1.6×10^7 cfu/ml

Footnotes: 20% CITMIT-DMDMH-EDTA constituted 0.00018% CITMIT, 0.044% DMDMH and 0.02% EDTA)

6.2.3.4 Preservative susceptibility of experimentally adapted isolates

The ancestral *P. aeruginosa* strain PA14 and adapted end-point populations from the planktonic and biofilm growth experiments were subjected to preservative susceptibility testing to determine the stability of phenotype. The isolates were assayed against CITMIT, DMDMH and the preservative combination CITMIT-DMDMH-EDTA. The MIC of CITMIT was the same for the ancestral *P. aeruginosa* PA14 and all of the end-point isolates, whilst the MIC for DMDMH and the CITMIT-DMDMH-EDTA combination was increased for the planktonic end-point populations that grew in 12.5% and 15% CITMIT-DMDMH-EDTA, and the biofilm end-point pooled growth from the TSB-only condition (Table 6.10).

Table 6.10 Preservative susceptibility of ancestral *P. aeruginosa* PA14 and derivative preservative combination-adapted populations.

Experimental conditions	Isolate/population	MIC (%)		
		CITMIT	DMDMH	CITMIT-DMDMH-EDTA
Ancestral strain	*P. aeruginosa* PA14	0.000625	0.0313-0.0625	7.5
Planktonic growth – end-point populations (one biological replicate tested)				
TSB only	TSB	0.000625	0.0313	7.5
TSB and 2.5% CITMIT-DMDMH-EDTA	2.5% CITMIT-DMDMH-EDTA	0.000625	0.0313	7.5
TSB and increasing CITMIT-DMDMH-EDTA	10% CITMIT-DMDMH-EDTA	0.000625	0.0625	7.5
	12.5% CITMIT-DMDMH-EDTA	0.000625	0.125	10-12.5
	15% CITMIT-DMDMH-EDTA	0.000625	0.125	10-12.5
Biofilm growth – end point populations (two biological replicates pooled and tested)				
TSB	TSB	0.000625	0.0625	7.5
TSB plus bead	TSB plus bead	0.000625	0.0313-0.125	7.5-10
TSB and increasing CITMIT-DMDMH-EDTA plus bead	20% CITMIT-DMDMH-EDTA	0.000625	0.0625	7.5

Footnotes: 100% strength CITMIT-DMDMH-EDTA constituted 0.0009% CITMIT, 0.22% DMDMH and 0.1% EDTA

6.3 Discussion

6.3.1 Preservative susceptibility of a diverse *P. aeruginosa* panel

Few studies have investigated the preservative susceptibility of *P. aeruginosa*. To further knowledge in this area, this chapter investigated a panel of *P. aeruginosa* strains for their susceptibilities to preservatives commonly used in HPC products. The strain panel comprised diverse strains from varied isolation sources including CF, CLIN, ENV and IND settings, with one strain also being of unknown origin. The MICs of six preservatives were determined against a panel of 40 strains and found to be highly variable, even between strains sharing the same AT-genotype, with the broadest variation in susceptibility observed for MIT, CITMIT, BIT and CHX. This was perhaps to be expected as biocide susceptibilities vary between microorganisms and strains of the same species (Maillard 2002; Rose *et al.* 2009; Rushton *et al.* 2013; Périamé *et al.* 2014) and the antibiotic and biocide susceptibility of *P. aeruginosa* strains has shown considerable variation (Chapman *et al.* 1998; Lambert *et al.* 2001; Van Eldere 2003; Lambert 2004).

None of the *P. aeruginosa* panel strains had a high tolerance for all of the preservatives that were investigated. Strains were identified that had high MIC values for the two preservatives of the same class (RW#138 for MIT and BIT, and RW#176 for MIT and CITMIT), with one of these strains also being highly tolerant to an unrelated preservative (RW#138 had the highest observed MIC for PHE). Interestingly, an elevated tolerance for one or two isothiazolinones did not correlate with a high tolerance to all three isothiazolinone preservatives; RW#176 had a slightly lower MIC for BIT, RW#138 had a low MIC for CITMIT, and RW#192 only had a high MIC for BIT. No strains were identified that had increased MICs for two or more preservative classes including CHX or BA. Overall, this suggests that different modes of entry/action and the resistance mechanisms are in operation for both different classes of preservatives and for preservatives within the same class. General resistance mechanisms for biocides are thought to include changes in outer membrane permeability and active efflux (Lambert *et al.* 2001), which could explain resistance to different compounds.

Whilst there are no recent systematic studies of biocide susceptibility for *P. aeruginosa* strain panels, the MIC ranges observed in this chapter are similar to previously published studies investigating *P. aeruginosa* susceptibility to MIT (0.0019%) (Winder *et al.* 2000), CITMIT (0.0003%) (Chapman *et al.* 1998), BIT (0.0056%; 0.0032%) (Winder *et al.* 2000; Abdel Malek and Badran 2010), PHE (0.25%) (Beveridge *et al.* 1980) and CHX (0.005%-0.02%; 0.0006%)

(Nakahara and Kozukue 1982; Abdel Malek and Badran 2010). Other studies have found MICs of preservatives approximately tenfold higher for CITMIT (Brozel and Cloete 1994) and BA (Borawska *et al.* 2008). In comparison to other species, the maximum preservative MICs for the panel of *P. aeruginosa* strains were slightly higher than those observed for *Burkholderia* species for MIT (0.00437%), CITMIT (0.000674%), BIT (0.008%) and PHE (0.25%) (Rushton *et al.* 2013) but lower than the highest MICs of CHX recorded (0.0039%; 0,01%) (Lambert *et al.* 2001; Rose *et al.* 2009). Maximum MICs previously documented for *E. gergoviae* strains were higher than the maximum recorded MICs for the *P. aeruginosa* panel for MIT (0.01%), but lower for CITMIT (0.000375%) and PHE (0.45%) (Périamé *et al.* 2014). Without the guarantee of standardised methods for MIC testing and statistical analyses, however, it is difficult to directly compare the results from other studies to the results in this chapter.

MIC values are clinically relevant as they are an important factor involved in deciding antibiotic treatment regimens (Lambert and Lambert 2006), and are obtained using standardised methods in clinical settings (Andrews 2001). The context of an MIC for preservatives can be debated, but in essence needs to fall below the maximal in use concentrations (see Table 1.2), and be proven effective in combination with other formulation components in HPC products. This chapter recorded MIC values using broth micro-dilution experiments involving planktonic growth in broth. MIC values will vary depending on whether solid or liquid media are used and whether planktonic or biofilm growth is investigated. These variables could be explored to further characterise the strain panel, in addition to performing minimum bactericidal concentration (MBC) experiments. Whereas MICs are the lowest concentration of an agent to inhibit visible overnight growth of a microorganism, the MBC of an agent represents the minimum concentration that will inhibit growth of an organism after subculture onto an antibiotic free medium (Andrews 2001), and the two frequently do not correlate (Langsrud and Sundheim 1997). Further investigations could also explore the susceptibilities of the *P. aeruginosa* strain panel to antibiotics, to determine if there is any correlation between antibiotic and preservative MIC values. Previous studies have been inconclusive, indicating both that highly antibiotic resistance Gram-negative bacteria also have a higher tolerance to disinfectants (Russell *et al.* 1986), and also that antibiotic resistance does not impact disinfectant resistance (Rutala *et al.* 1997). Potential cross-resistance of strains to antibiotics and biocides, and the emergence of antibiotic and biocide resistance, exacerbated by widespread biocide use, is a controversial topic still under investigation (Fraise 2002; Lambert 2004).

It should also be noted then when analysing MIC values, there appears to be no robust consensus method. The nature of MIC data is such that 'true' MIC values for strains within a

population lie on a continuum, but the recorded MIC values are discrete concentration increments (Lambert and Lambert 2006). Inherent problems in the documentation of MIC data include that the experimental methods and analyses may not be standardised between laboratories, that the discrete MIC data may not fit a normal distribution, and that small changes in the 'true' MIC could lead to a large change in the recorded MIC, depending on the concentration increments being used (Lambert 2004). It is therefore difficult to know whether statistical analyses should be performed for continuous (parametric) or discrete data (non-parametric). Frequently studies have used parametric methods which may not reflect the 'true' underlying mean and standard deviation values (Lambert and Lambert 2006). In an attempt to improve the interpretation MIC data, models have been proposed based on both cumulative frequency distributions (Lambert and Lambert 2006) and on survival analysis for interval censored data (van de Kassteele *et al.* 2012). Whilst these models better fit the nature of the MIC data, they are complex and not straightforward to implement (van de Kassteele *et al.* 2012). In this chapter, MIC data were analysed as discrete data with non-parametric statistics. The rationale behind this was that as the data were collected as discrete data, they should be analysed as such (Kowalski *et al.* 2005). Overall, this approach is less arbitrary than assigning mean values to discrete data categories and using parametric statistics, but less robust than using a statistical model.

6.3.2 Preservative susceptibility of industrial *P. aeruginosa* strains: potential impact on HPC products and industrial practices

The preservative susceptibilities of IND *P. aeruginosa* strains were compared to the susceptibilities of strains from CF, CLIN and ENV sources. For the isothiazolinone preservatives and PHE, the strains with the highest MIC values were from IND sources, posing the question whether stable adaptation to preservatives occurs in the industrial environment. This was also the case for CHX and BA, although maximum MIC values were shared between IND, CLIN and ENV strains. Whilst it appeared that overall, preservative susceptibilities of the IND strains were lower than all of the other strain groups, the median MIC value for IND strains was only determined to be significantly higher than the median MIC of the CF group for the preservatives MIT, CITMIT, BIT, PHE and BA. In addition the CLIN and ENV strains had significantly higher median MIC values than the CF strains for PHE. Although previous studies have found CF isolates to have high levels of antibiotic resistance (Breidenstein *et al.* 2011), little is known about their biocide resistance, and a number of strains in this chapter grew very slowly to low optical densities (5 strains grew to less than OD < 0.2 after 24 hours; Table 6.7) which would

have impacted the overall results. A larger strain panel would be needed to draw more meaningful conclusions about any link between isolation source and preservative susceptibility.

Preservative usage must be carefully balanced to prevent over-preservation, which can pose a risk of contact dermatitis to consumers, and under-preservation, which may result in product spoilage through microbial growth (Lundov *et al.* 2009). The maximum preservative concentrations permitted in HPC products are regulated in different regions to inform product formulation (Orth *et al.* 2006). In Europe, the EC Cosmetics Directive provides information concerning the type and quantity of preservatives that can be incorporated into different products (see Table 1.2). In this chapter five preservatives permitted for use in Europe were evaluated: MIT, CITMIT, PHE, CHX and BA. No preservative MICs above the maximum permitted levels were reported for the *P. aeruginosa* strain panel for MIT, CHX or BA. Seventeen of the panel strains, however, had MIC values for CITMIT that were above the EC maximum levels (0.0015%), in addition to one strain that exceeded the permitted level for PHE (1%). Whilst it is worrying that certain strains can grow in the presence of high levels of preservatives, MIC values in broth do not always reflect the performance of a preservative in product (Orth *et al.* 2006). In addition, a preservative system is typically composed of more than one preservative, preservative enhancing agents and other formulation components which interact to inhibit the proliferation of microorganisms (Orth *et al.* 2006). Therefore, to comprehensively evaluate the performance of a preservative, challenge testing with relevant strains should be carried out for the final product formulation.

Preservative efficacy testing or 'challenge testing' is performed by manufacturers to determine whether a product is adequately preserved to prevent microbial contamination. It involves the inoculation of product samples with indicator microorganisms, followed by the enumeration of survivors after stipulated time points in order to determine the reduction in viable cell numbers (Russell 2003). The microorganisms used in challenge testing can be from culture collections or are 'in-house' contaminants, and are chosen as likely product contaminants (Orus and Leranoz 2005). The *P. aeruginosa* panel investigated in this chapter included five strains which have been used in preservative efficacy testing (RW109, RW110, RW151, RW196 and RW198). These strains varied in their preservative susceptibilities, but did not have the highest observed preservative MICs for the panel, with the exception of RW110 for CHX. Based on the findings from the panel, the inclusion of strains with the highest tolerances for preservative agents (for example: RW138, RW146 and RW176 for the isothiazolinones; RW138 for PHE; RW110 for CHX; RW11 for BA) in challenge testing may be useful. It would have to be determined,

however, that strains with elevated MICs for individual preservatives had the same levels of resilience in complete products for the results to be valuable.

6.3.3 Anti-*P. aeruginosa* activity of preservative and preservative enhancer combinations

Combinations of preservatives are frequently used in HPC products to extend the spectrum of antimicrobial activity, and, if the combination exhibits additive or synergistic activity, boost the efficacy of microbial killing (Lundov *et al.* 2011). In addition, using preservative combinations often allows the levels of individual preservatives to be lowered, which decreases the risk of dose-dependent contact dermatitis (Lundov *et al.* 2011). Isothiazolinones are known contact sensitisers, with the chlorinated forms having higher potential for sensitisation (Alexander 2002), therefore a reduction in the concentrations used in products would be highly beneficial. This chapter looked at both the combination of two isothiazolinone preservatives (MIT and BIT) and an isothiazolinone preservative (CITMIT) with a potential preservative enhancer, the efflux pump inhibitor PAβN. This broad spectrum efflux pump inhibitor has been well documented and shown to reduce the MICs of several β-lactam antibiotics for Gram-negative bacteria including *P. aeruginosa*, allowing antibiotic accumulation within the cell (Mahamoud *et al.* 2007). Whilst the combination of MIT and BIT did not display enhanced antimicrobial activity for the 2 *P. aeruginosa* strains evaluated, the CITMIT and PAβN combination exhibited additive or synergistic activity for 9 out of the 10 *P. aeruginosa* strains tested. From these results it could be speculated that efflux of CITMIT plays a role in the tolerance of *P. aeruginosa* for this compound. Whether PAβN would be useful in industrial practices would need to be investigated further; the compound may not be stable in formulation and is relatively expensive, precluding its use.

The use of PHE has increased in the last 10 years, with figures from the US in 2007 stating it as the third most commonly used preservative (Lundov *et al.* 2011). As a preservative that is rarely associated with allergic contact dermatitis, PHE is a 'milder' preservation option, which appeals to increasing consumer demands for less harsh preservative systems in products (Varvaresou *et al.* 2009). PHE, however, is not quite as efficacious as preservatives such as the parabens, formaldehyde releasing agents and isothiazolinones (Lundov *et al.* 2009), and its activity may be enhanced in combination with other preservatives or preservative enhancing agents. In this chapter the combination of PHE with three potential preservative boosters was evaluated. The compounds CX3 and CX7 are cyclohexyloxyl derivatives with an unknown mechanism of action, and VSC (sorbitan caprylate) is a molecule with surfactant properties and is hypothesised to disrupt the cellular membrane (Pilz 2012). Only the combination of PHE with CX7 demonstrated

additive activity and would be potentially useful in products, but would require further evaluation. The combination of PHE with CX3 and VSC appeared to be antagonistic and would likely not enhance the preservative system within a product. This is in contrast to a previous study which found that the combination of PHE and VSC was synergistic (Pilz 2012), suggesting more work is needed to understand this particular preservative enhancer.

6.3.4 Phenotypic characteristics of a diverse *P. aeruginosa* panel

The growth curve dynamics and motility levels of the *P. aeruginosa* panel were very variable. By observing the 24 hour growth curve plots, the panel split largely into two groups; those that reached an OD over 0.20 after 15 hours, and those that did not reach 0.20 after 15 hours. In relation to isolation source, the majority of the IND, CLIN and ENV strains were found in the group growing at a higher OD, whilst the CF strains grew at a range. It is unknown if the OD was correlated to viable cell numbers or the biomass reached. The growth parameters lag phase, growth rate and maximum OD reached were analysed in more detail using statistical software, and no trends were observed between growth dynamics and isolation source. This is not conclusive evidence, however, as 7 strains had to be excluded from the analysis, and overall the strain panel was relatively small.

Motility was also very variable and there did not appear to be any link between motility levels and isolation source. It was expected that the CF strains within the panel would be less motile, as this is a characteristic of *P. aeruginosa* isolates from chronic lung infections (Mahenthiralingam *et al.* 1994), however this was not the case. As there were only 10 CF strains within a panel of only 40 strains, such differences may have been missed; the CF isolates were not representative of strains from chronic lung infection which are most likely to have adapted towards a non-motile phenotype. Furthermore, motility is dependent on growth media and conditions, illustrated by the fact that two of the panel strains, Pa#17 and Pa#18, have exhibited swarming motility in previous studies (Rashid and Kornberg 2000; Overhage *et al.* 2008; Tremblay and Deziel 2010), but were not observed to do so in this chapter. One interesting observation from the panel was the unusual swimming phenotypes demonstrated by the three IND strains from the same isolation source (RW130, RW131 and RW146). The cause of this phenotype is unknown but could suggest adaptation to that particular industrial environment.

6.3.5 Modelling adaptive resistance to a preservative combination

Adaptive resistance can be defined as the induction of often transient antimicrobial resistance in response to a certain stimulus (Fernández and Hancock 2012). The development of adaptive resistance to biocides has been observed for *P. aeruginosa* but the resistance mechanisms are poorly understood. Previous studies have examined adaptive resistance to the biocides CITMIT (Brozel and Cloete 1994), BIT (Al-Adham *et al.* 2012), PHE (Abdel Malek and Badran 2010), benzalkonium chloride (Joynson *et al.* 2002; Loughlin *et al.* 2002; Mc Cay *et al.* 2010), cetrimide (Al-Adham *et al.* 2012), didecyldimethylammonium bromide (Méchin *et al.* 1999), zinc pyrithione (Abdel Malek *et al.* 2009; Al-Adham *et al.* 2012), sodium pyrithione (Al-Adham *et al.* 2012) and monochloramine (Sanderson and Stewart 1997). Of these studies, which all investigated exposure to sub-inhibitory concentrations of a single biocidal agent, two used biofilm models (Sanderson and Stewart 1997; Al-Adham *et al.* 2012), whilst the others observed planktonic growth in broth containing increasing biocide concentrations.

Using *P. aeruginosa* PA14, a well-characterised, genome sequenced strain (O'Toole and Kolter 1998; Lee *et al.* 2006), and one of the most prevalent global clone types (Tümmler *et al.* 2014), this chapter examined the development of adaptive resistance using both planktonic growth and biofilm models. In addition, a combination of two preservatives (CITMIT and DMDMH) and one preservative enhancing agent (EDTA) was used, more reflective of preservative systems used in HPC products than a single preservative (Orth *et al.* 2006). In both models, adaptive resistance was developed to the CITMIT-DMDMH-EDTA combination; whilst growth of the ancestral PA14 was inhibited by 7.5-10% CITMIT-DMDMH-EDTA, end point PA14 derivatives of the planktonic growth and biofilm experiments could grow in 15% and 20% CITMIT-DMDMH-EDTA respectively. The end point PA14 derivatives were also re-tested for susceptibility to CITMIT, DMDMH and the CITMIT-DMDMH-EDTA combination. Isolates were found from both experiments with elevated MIC values for DMDMH and CITMIT-DMDMH-EDTA compared to the ancestral PA14 (Table 6.10). The long-term stability of the elevated resistance is unknown and would need to be investigated via progressive subculture in media without preservative, followed by further preservative susceptibility testing. No increases in MIC were observed for the isolates for CITMIT, however, which could be linked to the exposure level during the experiments being below the MIC of CITMIT for the ancestral PA14.

Biofilms are thought to be the preferred mode of bacterial growth in natural and industrial settings, and are frequently encountered in infection (Donlan and Costerton 2002). In industry, biofilm formation on industrial surfaces and machinery is very problematic, potentially leading

to product contamination and process inefficiency (Stickler 2004). Microorganisms adhered to industrial surfaces such as pipes are likely to be exposed a preservative concentration gradient, including potentially sub-lethal concentrations (Gilbert and McBain 2003). A biofilm model of adaptive resistance may therefore better represent preservative exposure in industry and was investigated in this chapter, utilising a recently published experimental evolution method (Poltak and Cooper 2011). This method was straightforward to set up and sample, and allowed the isolation of colony morphotypes linked to different stages of biofilm formation and adaptation (Figure 6.8 and Table 6.9). Furthermore, the difficulty of preservative neutralisation (Orth *et al.* 2006) prior to performing viable counts was negated, as biofilms adhered to beads were removed from the preservative solution and washed before sampling. Whilst these investigations were small scale, they have shown the applicability of the bead-biofilm model to examining adaptive resistance. Further work involving whole-genome resequencing of adapted isolates and transcriptomic analysis could be applied to the biofilm model to gain insight into genetic changes associated with adaptation to preservatives.

6.4 Conclusions

The main conclusions from the work in this chapter were as follows:

1) The preservative susceptibility of the *P. aeruginosa* panel was highly variable. There is a possibility that strains of IND origin have a higher tolerance to the isothiazolinone preservatives and PHE than strains from other isolation sources, and strains of CF origin have a lower overall tolerance to preservatives. Further investigation with a larger strain panel is required to confirm this.

2) The growth dynamics and motility of strains within the *P. aeruginosa* strain panel were also very variable and motility did not appear to be linked to isolation source. These findings are in concordance with the theory that *P. aeruginosa* isolates from different environments are functionally indistinguishable from one another, but further work with a larger strain panel is required to obtain more conclusive results.

3) The combination of CITMIT and the efflux pump inhibitor PAβN displayed the most efficacious anti-*P. aeruginosa* activity, having an additive or synergistic interaction for 9 out of the 10 *P. aeruginosa* strains tested. It can also be hypothesised from these findings that efflux is involved in the resistance of *P. aeruginosa* to isothiazolinone preservatives.

4) The development of adaptive resistance to a preservative combination was observed in both planktonic growth and biofilm models, although the stability of the resistance is unknown. The biofilm model of adaptive resistance has potential for further development to investigate adaptation to preservatives used in industry.

The hypotheses proposed at the beginning of this chapter were accepted:

Hypothesis 1: The phenotypic characteristics of *P. aeruginosa* strains are highly variable and unlinked to habitat

Hypothesis 2: Adaptive resistance can be developed to a preservative combination in planktonic and biofilm growth models, where *P. aeruginosa* can grow in concentrations of the preservative combination above the MIC level for the ancestral strain.

7. General conclusions, discussion and future research

7.1 Conclusions

A novel and comprehensive study of *P. aeruginosa* as a bacterial contaminant of HPC products was conducted. Both culture-dependent and culture-independent methods for the detection of the species from HPC products were investigated, and the applicability of the detection methods to industrial practices considered. The strain diversity of *P. aeruginosa* in industry was examined and related to the overall population biology of the species. Furthermore, the bacterial diversity encountered in HPC products was assessed to determine the species associated with HPC product contamination incidents. In addition, a panel of *P. aeruginosa* strains from diverse isolation sources were characterised for their susceptibility to commonly used HPC preservatives, growth and motility characteristics, to determine any links between isolation source and phenotype. Finally, the evolution of resistance to a preservative combination was investigated using both planktonic growth and biofilm models.

The main conclusions from the study are as follows:

1. **Culture-dependent detection of *P. aeruginosa* in industry can be complemented by culture-independent detection methods involving direct DNA extraction from HPC products and the application of species-specific PCRs (Chapter 3 and 4).**

Culture-dependent methods are used routinely in industry to detect bacterial contaminants but can lack selectivity, may introduce a culture bias and neglect the detection of VBNC microorganisms. Selective agars containing cetrimide as the selective agent are recommended for the detection of *P. aeruginosa* from HPC products. Whilst the comparative agar study conducted in Chapter 3 demonstrated that the cetrimide-based agar PCN performed the best overall, the selectivity of the agar was relatively low (34% after 24 hours incubation at 37°C). Few studies have extracted nucleic acids directly from HPC products without a prior culture enrichment step. The work in Chapter 4, however, demonstrated that DNA may be extracted from a variety of contaminated HPC product types using a standardised and automated system: the Maxwell®16 instrument and tissue DNA extraction kits.

A novel species-specific PCR for *P. aeruginosa* using *phzS* as the target gene was designed and evaluated using both a strain panel and contaminated HPC products. The *phzS* PCR showed potential as a new molecular diagnostic for *P. aeruginosa*, having 100% sensitivity and

specificity. The *phzS* PCR also matched the performance of the widely used *oprL* gene PCR (De Vos *et al.* 1997). Application of species-specific PCRs could improve the identification of contaminants, particularly those which are difficult to cultivate or distinguish from mixtures of contaminants where colony morphologies are similar. Further work is needed to more accurately determine detection limits of the *phzS* PCR. The use of HPC product samples artificially spiked with known numbers of cells and the development of a *phzS* qPCR could facilitate this.

2. **The bacterial diversity associated with HPC products is low and a combination of both culture-independent and culture-independent approaches gives the most comprehensive assessment of contaminating species (Chapter 4).**

This was the first study to use both culture-dependent and –independent techniques to assess the bacterial diversity associated with contaminated HPC products. Overall, bacterial diversity was low and contamination was largely due to one dominant genus, with no more than 3 genera being observed in total (*Pseudomonas*, *Enterobacter* and *Herbaspirillum*). This corroborated previous findings from studies of product recall data (Wong *et al.* 2000; Jimenez 2007; Lundov and Zachariae 2008; Sutton and Jimenez 2012), but was a much more thorough investigation, using a 16S rRNA gene metataxonomic sequencing approach. Limitations with both culture-dependent and culture-independent approaches, including issues with product neutralisation and the presence of potential PCR inhibitors, indicated that a combination of both approaches was necessary to fully catalogue the bacterial diversity present.

3. ***P. aeruginosa* has a non-clonal epidemic population structure and industrial *P. aeruginosa* strains are genotypically and phenotypically similar to strains from cystic fibrosis, clinical and environmental sources (Chapter 5 and 6).**

The strain diversity and population biology of *P. aeruginosa* in industry have not previously been investigated. Using five genotyping techniques (RAPD, AT-typing, MLST, rMLST and wgMLST) the work in Chapter 5 determined that *P. aeruginosa* strains from industrial sources were diverse and that there was no association between strain type and HPC product type. In addition, industrial strains were a mixture of novel and previously found genotypes, and the previously found genotypes were linked to multiple habitats. AT-typing and eBURST analysis of a database containing 941 *P. aeruginosa* strains demonstrated that overall the *P. aeruginosa* population structure was non-clonal epidemic. Only 1.7% of the database strains originated from industry, however, indicating the need for a strain panel which is more balanced with

regards to isolation source. In general, no major phenotypic differences were identified between strains from cystic fibrosis, clinical, environmental or industrial sources (Chapter 6), further corroborating the lack of habitat selection displayed by *P. aeruginosa*. Whilst the preservative susceptibilities of the industrial strains appeared lower than the rest of the panel, this was not shown to be statistically significant. In addition, the growth dynamics and motility characteristics were highly variable across the whole panel of *P. aeruginosa* isolates.

4. **Bacterial genotyping methods may be of use to industry to identify persistent strains within the industrial environment (Chapter 5).**

Where bacterial strains have been cultivated from industrial products or from the industrial environment, genotyping techniques can be used to identify problematic or recurrent strain types. In this study three strains isolated from contaminated DWL in Italy were found to have the same genotype, despite being collected 6 years apart; RW130 and RW131 were isolated in 2010, and RW146 in 2004. This indicates persistence of the strain type in the industrial environment which may be linked to recurrent contamination of products. Interestingly, although the same AT-genotype and ST, the three strains had varying preservative susceptibilities. The growth curve parameters for the strains could not be determined but all three strains reached a similar culture density by 15 hours (Figure 6.3), had similar swimming and swarming characteristics (Table 6.8), and did not display twitching motility. In addition, the strains had an atypical swimming motility morphology (Figure 6.6) not seen for other panel strains, suggesting potential adaptation to the industrial environment.

5. **Evolution of adaptive resistance to a preservative combination can be achieved using both planktonic growth and biofilm models. Further development of the biofilm model could prove useful in understanding the development of adaptive resistance in industrial settings (Chapter 6).**

The development of adaptive resistance to preservatives is poorly understood and the majority of previous studies have looked at planktonic growth in the presence of a single preservative. To more accurate model preservative adaptation, this study examined planktonic and biofilm growth and used a preservative combination, as would be found in HPC products. *P. aeruginosa* grew in elevated levels of preservatives in both models. Whether the increased tolerance was stable needs to be further investigated, but this preliminary work has suggested that the biofilm bead model (Traverse *et al.* 2013) is applicable to studying the evolution of preservative resistance.

7.2 Discussion and future research

In the majority of scientific literature, *P. aeruginosa* is most commonly recognised as an opportunistic pathogen associated with the nosocomial environment. As such, relatively little is known about *P. aeruginosa* in environmental niches or industrial settings. In addition, whilst the resistance of *P. aeruginosa* to antibiotics is well documented, the mechanisms of preservative resistance are poorly understood. This study has provided insight into *P. aeruginosa* in industry, focussing on the detection, diversity and genotypic and phenotypic characterisation of strains found as contaminants of HPC products. In this section, the results of the study will be discussed with reference to industrial practices and future directions will be suggested.

7.2.1 *P. aeruginosa* in industry

7.2.1.1 Can the detection of *P. aeruginosa* in HPC products be improved?

The detection of *P. aeruginosa* and other microbial contaminants of HPC products is currently largely reliant on culture-dependent methods, the limitations of which have been previously discussed. The development of new selective media for the improved detection of *P. aeruginosa* is unlikely; the only novel media developed for this purpose in the last decade (chromID Pa) (Laine *et al.* 2009) was demonstrated in Chapter 3 to have inferior sensitivity to the widely used cetrimide-based agar PCN. Alternative and more recently developed, 'rapid' methods such as ATP-bioluminescence, impedance and flow cytometry are available (Jimenez 2001b), but estimate total bioburden rather than identifying specific species. The inclusion of culture-independent methods in routine contaminant detection procedures would therefore benefit industry, affording increased detection sensitivity and specificity. With the optimisation of DNA extraction and PCR protocols, it is also likely that the detection limits could drop below 10^2 cfu/g of product.

The implementation of such procedures may be challenging, however, as the introduction of a new process into a company has many considerations. In the instance of contaminant detection in non-sterile products, the guidelines of professional organisations such as the PCPC, FDA, SCCS and Cosmetics Europe (see section 1.1.2), and any specified in-house standards, must be taken into account to ensure product quality and safety. Furthermore, multi-national companies have to consider standardisation and harmonisation of new processes, which aims to achieve results that are comparable between different regions (Tate and Panteghini 2007). If culture-

independent detection methods were deemed suitable for contaminant identification, their company-wide implementation would require a huge concerted effort. Considerations would include, but not be limited to, overall costings, equipment and reagent purchases, equipment maintenance, theoretical and technical training for personnel, the development of standard operating procedures (SOPs) for personnel and risk assessment documentation.

7.2.1.2 Sources of contamination in industrial settings

The manufacture of HPC products is not conducted in a sterile environment, and this combined with the large scale of production provides numerous opportunities for product contamination to occur. Whilst good manufacturing practices (GMPs) are strictly followed to reduce microbial contamination (Perry 2001), it is not possible to eliminate the risk completely. Contamination may arise from raw materials or the manufacturing environment, where the design of the process equipment and plant cleanliness will contribute to the microbial bioburden found in finished products (Maukonen *et al.* 2003). Water is used extensively in manufacture, both as a raw material and to rinse and clean equipment, floors and walls (Jimenez 2007), and is a potentially major source of microbial contamination. Gram-negative genera including *Pseudomonas*, *Burkholderia*, *Alcaligenes*, *Stenotrophomonas*, *Ralstonia*, *Serratia* and *Flavobacterium* are commonly found in water samples and may form biofilms in water storage and distribution systems (Jimenez 2007). Certain points within piping systems, such as dead-ends, corners, cracks, and joints, are conducive to the formation of biofilms, which can be difficult to eradicate and may act as a reservoir for microorganisms (Maukonen *et al.* 2003). It is therefore important that sampling valves are positioned throughout the system to monitor water quality, and effective decontamination measures are in place (Maukonen *et al.* 2003; Kawai *et al.* 2004).

7.2.1.3 Is the industrial environment an important niche for *P. aeruginosa*?

P. aeruginosa is postulated to be a predominantly aquatic microorganism in the natural environment (Selezska *et al.* 2012) and has minimal nutritional requirements which facilitate its survival in distilled water (Favero *et al.* 1971; Kayser *et al.* 1975). Together with a propensity to form biofilms, these characteristics make *P. aeruginosa* a likely contaminant of industrial water systems and raw materials with high water content. It is possible that *P. aeruginosa* is present throughout industrial manufacturing systems, but no studies have sampled this environment specifically for *P. aeruginosa*. Whilst this study (Chapter 5) demonstrated that industrial strains

were diverse and there was no link between strain type and product type, only contaminants isolated from HPC product samples were surveyed.

Further questions remain therefore, including: (i) what is the diversity of *P. aeruginosa* strains associated with industrial manufacturing sites, and how does this relate to global *P. aeruginosa* diversity? (ii) is the diversity equivalent to that found in industrial products, or is there a sub-group of *P. aeruginosa* strains selected for in products? (iii) what are the main sources of bacterial contamination in industry? An approach such as that of Quick *et al.* (Quick *et al.* 2014) could be applied to industrial settings to investigate this. Quick *et al.* used WGS and metagenomics to trace the source of *P. aeruginosa* infections in burn victims at a recently opened hospital. Genomes of 144 *P. aeruginosa* isolates from patients, the hospital environment, water outlets and water samples were sequenced, in addition to the metagenome of a biofilm recovered from a thermostatic mixer valve. Phylogenetic analysis of study isolates and a global strain collection identified 8 distinct clades but found no overt association between genotype and ecological setting. In addition, genotypes of isolates recovered from patients were linked to isolates from water outlets in their rooms, suggesting transmission from the hospital water system.

In industry, this study design would translate to sampling different points within manufacturing environments, water distribution systems, raw materials and finished products. The genomes of recovered isolates could then be compared to each other and also combined with a data from a global collection of *P. aeruginosa* strains, as performed by Quick *et al.* (Quick *et al.* 2014). Large-scale sequencing projects such as this can provide unparalleled insight into the epidemiology and population biology of *P. aeruginosa*, but would benefit from balanced numbers of isolates from different sources (Spratt and Maiden 1999), including industry, to provide a more balanced view of population structure.

7.2.2 *P. aeruginosa* resistance to preservatives

7.2.2.1 Improving the understanding of preservative resistance

P. aeruginosa is well documented to have resistance to multiple classes of antimicrobials (Poole 2011), including preservatives (McDonnell and Russell 1999). In contrast to antibiotics, which have well defined targets within bacterial cells, preservative modes of action are poorly understood although believed to involve multiple cellular target sites (Maillard 2002). Similarly

the preservative resistance mechanisms employed by bacteria are not well characterised. It is likely, however, that both intrinsic mechanisms, such as low overall outer membrane permeability and efflux systems (see section 1.3.2), and adaptive resistance mechanisms including biofilm formation (see section 1.3.4 and 1.3.5) are important in *P. aeruginosa* (McDonnell and Russell 1999).

In industry, the exposure of microorganisms to sub-lethal concentrations of preservatives may trigger the development of adaptive resistance (Jimenez 2007). Adaptive resistance mechanisms for *P. aeruginosa* have been identified as generally nonspecific, involving changes in outer membrane proteins (Brozel and Cloete 1994; Abdel Malek *et al.* 2009; Abdel Malek and Badran 2010), fatty acid profiles of membranes (Jones *et al.* 1988; Méchin *et al.* 1999; Loughlin *et al.* 2002) and increased efflux (Abdel Malek *et al.* 2009). Adaptive resistance to a preservative combination was observed in this study (Chapter 6) using both planktonic and biofilm growth models. It is unknown whether this resistance was stable, whether adapted derivatives displayed cross-resistance to other antimicrobial agents, and what the underlying molecular mechanisms were, warranting further research in this area.

To gain a greater understanding of the processes involved in the development of adaptive resistance, further work with the planktonic and biofilm models used in Chapter 6 could be performed. Experimental evolution models such as these are controlled and replicable, and allow changes in fitness can be tracked directly through sampling at different time points (Dettman *et al.* 2012). When these experiments are combined with WGS, genome sequences of ancestral, intermediate and evolved strains can provide a record of the genetic changes which have occurred over the course of the experiment (Dettman *et al.* 2012). Whilst this has not been used for preservative adaptation yet, it has been used for *P. aeruginosa* adaptation to antibiotics (Wong *et al.* 2012), and for biofilm formation in *Burkholderia* (Poltak and Cooper 2011). Comparisons between genome sequences of an ancestral *P. aeruginosa* strain and derivatives grown in the presence of preservatives could reveal mutations underlying adaptation.

It is also likely that changes in gene expression in response to preservative exposure are important in the development of adaptive resistance. Transcriptomic studies examine gene expression on a global scale, and can provide a 'snapshot' of a microorganisms's response to variations in environmental conditions (Balasubramanian and Mathee 2009). Detection and quantification of RNA transcripts has been performed for *P. aeruginosa* under numerous conditions including iron limitation, oxidative stress, osmotic stress, biofilm formation and interactions with human cells (Balasubramanian and Mathee 2009), although no studies have

yet looked at preservative exposure. Transcriptomic analysis using DNA microarray technology has been used to examine the molecular basis for adaptive resistance to CITMIT in *B. lata*, however, identifying altered expression of multiple genes involved in transport and highlighting efflux as an important resistance mechanism (Rushton *et al.* 2013). Interestingly, in this study the adaptation of *Burkholderia* to CITMIT was associated with a stably resistant phenotype and permanent changes in gene expression (Rushton *et al.* 2013). A similar approach could be taken for *P. aeruginosa* exposure to the preservative combination used in this study, although taking advantage of RNA-seq as an improved method of transcriptional analysis (Croucher and Thomson 2010).

7.2.2.2 The impact of understanding preservative resistance on industry

Understanding how *P. aeruginosa* responds to stresses such as preservative exposure will lead to a better understanding of its success as an industrial contaminant, and inform preservation strategies. Experimental evolution models have the potential to highlight preservatives which bacteria can develop adaptive resistance to, and whether the adaptive resistance is stable. Much is currently unknown about the longevity of adaptive resistance (Fernández *et al.* 2011), with different studies reporting both transient (Brozel and Cloete 1994) and stable (Winder *et al.* 2000; Rushton *et al.* 2013) increases in resistance in bacteria on exposure to sub-inhibitory preservative levels. Whilst the levels of preservatives in products are generally high enough to prevent growth or the development of adapted strains, exposure to sub-inhibitory levels of preservatives within the manufacturing environment is a possibility (Orus and Leranoz 2005). In addition, the persistence and recurrent contamination caused by a single *P. aeruginosa* strain type at an HPC manufacturing site (Chapter 5), is suggestive of the fact that strains can show an elevated fitness in the industrial environment, although in this instance the strain involved did not show specific preservative resistance adaptations. It may therefore be beneficial to include adapted bacterial strains in routine preservative efficacy testing (see section 1.1.2) strain panels for HPC products, as stably adapted strains may display higher levels of preservative resistance, increasing the stringency of the challenge testing procedure.

Where different antimicrobials act upon the same cellular target or cellular pathways, there is the potential for cross-resistance to develop (Chapman 2003b). As preservative mechanisms of action are considered to be general and bacterial preservative resistance mechanisms thought to be non-specific, the development of cross-resistance between preservatives and other antimicrobial classes is of concern (Russell 2000). For *P. aeruginosa*, adaptive resistance to biocides has been linked with an increased tolerance to other classes of biocides and antibiotics

(Adair *et al.* 1969; Jones *et al.* 1988; Loughlin *et al.* 2002; Langsrud *et al.* 2003; Abdel Malek *et al.* 2009; Abdel Malek and Badran 2010). In industry, this information could inform the development of preservative systems, which usually comprise two or more compounds with different modes of action (Orus and Leranoz 2005), to combat the emergence of resistant strains in products (Orth *et al.* 2006). Furthermore, the rotation of biocides used as disinfectants in industrial settings is supported (Langsrud *et al.* 2003), but if cross-resistance is occurring then the efficacy of the regimen will be compromised. Determining which biocides are affected by this phenomenon would improve industrial practices.

Identifying resistance mechanisms may also further help with product formulation through the selection of appropriate preservative enhancing agents. For example, the inclusion of chelating agents such as EDTA in formulations increases the permeability of Gram-negative cell membranes, potentiating the activity of numerous classes of antimicrobial agents. The study by Rushton *et al.* (Rushton *et al.* 2013) demonstrated an increase in the susceptibility of *B. lata* to the isothiazolinone preservative CITMIT in the presence of the efflux pump inhibitor PaβN. This PhD (Chapter 6) also found that in 9 out of the 10 *P. aeruginosa* strains tested, there was an additive/synergistic relationship between CITMIT and PaβN. These results suggest that efflux may be involved in resistance to CITMIT. The inhibition of microbial efflux may therefore be a valid strategy to improve the performance of certain antimicrobial agents (Tegos *et al.* 2011). The stability of efflux pump inhibitors in product formulations, however, would also have to be considered for use in the HPC industry.

7.3 Perspectives

This study has re-enforced the fact that *P. aeruginosa* is a truly ubiquitous microorganism. The association of the species with water poses significant problems for the HPC industry where water is an important raw material and used in plant cleaning operations. Owing to its metabolic versatility, high levels of antimicrobial resistance and ability to form biofilms, *P. aeruginosa* would be extremely difficult to eliminate from the industrial environment. Improved methods for the detection of *P. aeruginosa*, both from water distribution systems and HPC products, would therefore be beneficial to manufacturers. As it is very difficult to implement new procedures within a multinational company, culture-independent detection methods could be trialled to complement, rather than replace, current culture-dependent practices. Furthermore, the application of WGS to bacterial isolates from industry, as has already been performed in the nosocomial environment, could highlight sources of contamination and further investigate *P. aeruginosa* population biology. Finally, continued research into the preservative resistance mechanisms of *P. aeruginosa* has the potential to inform the design of preservative systems and efficacious factory decontamination measures.

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