Molecular Detection and Characteristics of *Burkholderia Cepacia* Complex Strains Isolated as Industrial Contaminants

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Abstract

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Anjeet Jhutty

During manufacture, bacteria that persist in the environment or are present in raw materials can contaminate consumer products. Current QC methods rely on slow and error prone microbiology techniques resulting in QC re-testing and product recalls. Preservative agents are added to formulations to ensure stability and sterility throughout the product life cycle. Despite this, certain bacteria, such as those belonging to the Burkholderia Cepacia Complex (Bcc), can cause product spoilage even with a preservation strategy. In response to growing bacterial preservation resistance coupled with heightened restrictions on conventional preservative agents and an increase in consumer demand for greener preservation systems, research is moving towards natural alternatives.

Molecular methods for bacterial detection in personal care products was investigated. Development of a broad range bacterial detection methodology was attempted however a high-resolution broad range molecular detection method could not be constructed within the parameters required for routine QC microbiology. An assay for rapid bacterial detection and identification within potentially contaminated personal care products was developed. This assay was performable in 7 hours, had a detection sensitivity of 10^3 CFU/ml and identified bacterial DNA in archived contamination samples which did not produce viable counts. However, identification using species specific nested primers was not successful making its utility limited.

Preservative susceptibility of 47 *Burkholderia* strains was examined using nature identical preservatives and synthetic preservatives. Organic acids: Benzoic acid and salicylic acid required in-use concentrations comparable or lower than DMDMH and similar to methyl paraben. Closer examination of the nature identical preservatives benzyl alcohol, phenoxyethanol, salicylic acid and benzoic acid with four Bcc strains highlighted variability in preservative activity with the organic acid preservatives. Nature identical preservatives were able to inhibit genomically diverse *Burkholderia* strains at concentrations that did not exceed the maximum permitted amounts. This illustrates the activity of greener nature identical preservatives exceeds or is comparable to some commonly used synthetic agents.

Adaptive bacterial resistance to nature identical preservatives benzyl alcohol and phenoxyethanol plus the synthetic preservative BIT was examined using four contaminant Bcc strains and two methods: high concentration/fast exposure timescale and low concentration/slow exposure timescale. Stable adaptive resistance was induced with phenoxyethanol and BIT but not with benzyl alcohol. Preservative dose/timescale did not affect the induction or stability of adaptive resistance phenotype but was associated with a lower level of susceptibility.

The genomic basis of preservative adaptive resistance in Bcc strains was examined through sequencing, variant calling and GO enrichment analysis. Stable adaptive resistance gene variants were mostly associated with metabolism. Variants observed in preservative exposed Bcc strains indicated the significant involvement of stress resistance mechanisms and enzymatic degradation. GO enrichment highlighted the involvement of xenobiotic degradation. This indicates preservative adaptive resistance in Bcc strains is mediated through indirect resistance mechanism which enable survival and mitigate damage.

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List of Abbreviations

AHLs	N-acyl homoserine lactones
AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BBSRC	Biotechnology and Biological Sciences Research Council
BCC	*Burkholderia cepacia* complex
BIT	Benzisothiazolinone
BSM	Basal salt medium
CASE	Collaborative Awards in Science and Engineering
CDC	Centers for Disease Control and Prevention
cDNA	Complementary Deoxyribonucleic acid
CF	Cystic Fibrosis
CFTA	Cosmetic, Toiletry and Fragrance Association
CFU	Colony Forming Unit
CGR	Centre for Genomic Research
CMIT	Chloromethylisothiazolinone
DMDMH	Dimethylol Dimethyl Hydantoin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
EEC	European Economic Community
EEC	European Economic Community
EMA	Ethidium monoazide
EP	European Pharmacopeia
EPS	Extracellular polysaccharides
FDA	Food and Drug Administration
FS	FisherStrand
FTM	Fast and Transient Mechanisms
GATK	Genome Analysis Tool Kit
gDNA	Genomic deoxyribonucleic acid
H/F	High Concentration and Fast Timescale
HAQs	Hydroxy-2-alkylquinolines
HMAQs	Hydroxy-3- methyl -2-alkylquinolines
ICU	Intensive Care Unit
JP	Japanese Pharmacopeia
KDO	3-deoxy-D-manno-2-octulosonate
L/S	Low Concentration and Slow Timescale
LB/LA	Luria Broth/Agar
LPS	Lipopolysaccharides
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MDR	Multidrug resistance
MIC	Minimum Inhibitory Concentration
MIT	Methylisothiazolone
ml	Millilitre
MQ	RMSMappingQuality
MQRankSum	MappingQualityRankSumTest

mRNA	Messenger Ribonucleic acid
MRSA	Methicillin-resistant Staphylococcus aureus
MSTL	Multi Locus Sequence Typing
OD	Optical Density
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type
ng	Nano gram
PacBio	Pacific Biosciences
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate-buffered saline
PCBs	Polychlorinated biphenyls
PCR	Polymerase Chain Reaction
PDR	Pan-drug resistance
PET	Preservation Efficacy Test
PFGE	Pulsed-Field Gel Electrophoresis
PMF	Proton Motif Force
QA	Quality Assurance
QAC	Quarternary ammonium compounds
QC	Quality Control
QD	QualByDepth
qPCR	Quantitative Polymerase Chain Reaction
QS	Quorum Sensing
R&D	Research and Development
R2A	Reasoner's 2A
RDP	Ribosomal Database Project
ReadPosRankSum	ReadPosRankSumTest
RFLP	Restriction Fragment Length Polymorphism
RMM	Rapid Microbiological Methods
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPM	Rotations Per Minute
rRNA	Ribosomal Ribonucleic acid
SDA PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
ssDNA	Single stranded deoxyribonucleic acid
SSM	Slow and Stable Mechanisms
TAMC	Total Aerobic Microbial count
Taq	*Thermus aquaticus*
TCS	Two-component regulatory systems
TMM	Traditional Microbiological Methods
TSA	Tryptone Soy Agar
TSB/TSA	Tryptone Soy Broth/Agar
TVC	Total Viable Count
TYMC	Total Yeasts and Moulds count
USP	United States Pharmacopeia
UV	Ultraviolet
V/V	Volume by Volume
VBNC	Viable But Not Culturable
W/V	Weight by Volume
XDR	Extensively drug resistant

Chapter 1: Introduction

1.1 Microbial Spoilage in the Manufacture of Personal Care Products

1.1.1 Current Pharmacopeial Guidelines

All manufacturers have a legal duty and ethical responsibility to ensure products are manufactured and maintained throughout their lifecycle in a hygienically safe condition. Pharmacopeia are country specific regulatory guidelines for pharmaceutical products, which include strict specifications on quality control (QC) and quality assurance (QA) measures. Pharmacopeial standards are used in the examination of non-sterile topical and oral preparations such as cosmetics, shampoos, body lotion, sun protection, facewash, toothpaste and mouthwash (Ratajczak *et al.*, 2015). Thirty years ago, countries followed national pharmacopeial guidelines, i.e. British Pharmacopeia, however with the need for greater clarity and consensus of quality standards across countries, three main pharmacopeial standards were founded: European Pharmacopeia (EP) and United States Pharmacopeia (USP) and Japanese Pharmacopeia (JP) (Sutton, 2006). Between 2007-2010 European, United States and Japanese pharmacopeia, methodologies were standardised across the three-reference works (in a process known as harmonisation) allowing products manufactured for international distribution to avoid repeat testing and redundant microbiology examinations to meet continent specific specifications.

There are strict guidelines as outlined in pharmacopeia limiting bioburden (the number of micro-organisms in a non-sterilised product) and strains of acceptable microorganisms found in non-sterile pharmaceutical formulations (Sutton, 2006). This is broadly categorised into three main areas: Total Microbial Enumeration (EP Harm 2.6.12/USP Harm 61/ JP 15th edition 1st Supplement), Absence of Specified Microorganisms (EP Harm 2.6.13/USP Harm 62/ JP XV 1st Supplement) and Microbiological Attributes of Non-Sterile Pharmaceutical Products (EP Harm 5.1.14/USP Harm 1111/ JP 15th edition 1st Supplement-General information). Product quality assessments can be performed using traditional microbiological methods (TMM) using culture based biochemical and morphological assessments, or

using Rapid Microbiological Methods (RMM), which can be culture or non-culture based, but are significantly quicker than traditional methods and are often automated (Scott, 2013).

The Total Microbial Enumeration test is a count of the total number of viable aerobic micro-flora present in a non-sterile finished product or raw material. This is primarily executed using traditional plate count methods, which calculate colony-forming units (CFUs) by serial dilution and direct plating. Results take an average of 3-5 days (Pharmacopoeia, 2016a). In order to assist in the recovery of micro-organisms a 30 min incubation period with neutralising peptone buffers (typically 0.1% peptone and 2% tween 80) is used to counteract the presence of anti-microbial preservatives in formulations (Amezaga and Booth, 1999). Samples are plated out onto Tryptone Soy Agar (TSA), a nutrient rich medium able to cultivate a broad range of microorganisms.

In order to increase sensitivity and speed of Total Microbial Enumeration tests, a rapid biochemical method was adapted for use in QC microbiology. ATP bioluminescence assays exploit the natural chemical conversion of energy to light which occurs in all living organisms. In the luciferin/luciferase bioluminescence reaction metabolism of cellular ATP results in the omission of yellow-green light (550 - 570nm). ATP is found in all viable cells, making it a reliable biomarker for microbial contamination (Chollet and Ribault, 2012).This can be detected and even semi-quantified as bioluminescence events are proportional to the presence of bacterial cells (Prince, 2008). The Celsis RapiScreen is the most popular commercially developed ATP bioluminescence platform for pharmaceutical use. Celsis assays increase the sensitivity and speed of bacterial detection and can provide results in 24-48 hours (Charles River Laboratories Inc, 2016).

The Absence of Specified Micro-organisms test confirms the absence or limited occurrence of 6 specified aerobic/facultative organisms, *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027) *Escherichia coli* (ATCC 8739), *Salmonella typhimurium* (ATCC 14028) or *Salmonella abony* (NCTC 6017), *Candida albicans* (ATCC 10231) and the anaerobic organism *Clostridium sporogenes* (ATCC 11437). This is formulation dependent, and most products do not

have to demonstrate the absence of the entire panel (European Pharmacopoeia, 2016b)

The Microbiological Attributes of Non-Sterile Pharmaceutical Products is a new addition to the EP and USP guidelines, brought in at the time of harmonisation in 2007. These guidelines suggest other objectionable organisms that indicate microbial quality such as process contaminants like *Burkholderia cepacia* complex strains (Bcc) (Gordon *et al*., 2011). The absence of specified organisms is traditionally done using biochemical assays and morphological assessment on selective media, which can take up to 7 days to obtain a result (European Pharmacopoeia, 2016c).

When a pharmaceutical non-sterile product fails any stage of the QC examination, a species level identification is required in order to determine what action to take. Identification of contaminating microorganisms relies on standard microbiological culture techniques and can take up to 5 days. In some cases, harvesting viable colonies from contaminated personal care products is not possible at all. If colonies are able to be harvested, a series of biochemical tests (48 hours), selective media streaks (72 hours) and DNA sequencing methods (48 hours) are used to give a robust identification. Modern production management systems require faster identification for a quicker response to contamination events, preferably giving results within one working day or a ‘7 hour shift’ (Denise Donoghue, personal communication).

1.1.2 Consequences of Microbial Contamination

The most significant problem with the current pharmacopeial methods for microbiological QC is their dependency upon culture techniques for the identification and characterization of microorganisms. These cultivation-based methods are ideal for microflora present in high numbers and almost pure culture but are ineffective at identifying very low levels of total bioburden in semi-viscous pharmaceutical non-sterile products (Denyer, 2006).

Total viable counts are known to cause underestimates of total bioburden, due in part to spreader impaction injury to cells, which can cause a 20% reduction in CFU counts for gram-negative Proteobacteria (Thomas *et al.*, 2014). Variability in viable counts can also be caused by sampling errors and the residual antimicrobial effect of

pharmaceutical preservatives (Baird *et al*., 2005). Total viable counts performed on artificially spiked shampoo greatly under-represented bacterial bioburden by up to 1000-fold (Jhutty, unpublished).

Organisms may suffer from nutrient shock effects when adapting from a low nutrient consumer product to a nutrient rich medium resulting in retarded growth and false negative QC results (Torbeck *et al.*, 2011). Furthermore, many organisms can enter a viable but non culturable (VBNC) state, whereby cells reduce growth and metabolic activity in unfavourable conditions but are alive and once resuscitated can resume normal functions (Oliver, 2005). Furthermore, these methods are laborious, prone to contamination, time consuming (average of 3-10 days for results) and are not always sufficiently specific to discriminate between species and strains (Baird *et al*., 2005).

Rapid molecular methods such as ATP bioluminescence can be used to speed up results. However, these methods cannot be used on formulations with natural ingredients as non-microbial ATP is also detected and are only semi-quantitative. VBNC organisms can remain undetected and identification methods are needed as this method is not discriminatory. The ATP bioluminescence method is also limited by the pH, where the optimal value for detection 7.5 to 7.8 (Wang *et al*., 2013).

Despite their disadvantages, cultivation-based techniques for enumerating bacteria are often chosen over alternative methods because they are cheap, simple to perform, and high throughput (Sutton and Luis, 2012).

Aside from the disease-causing potential of contaminated personal care products (discussed in detail in section 1.2), contaminated pharmaceutical consumer products can have severe financial impacts for manufactures. Product spoilage subsequently leads to product recalls, which aside from the direct costs involved from physical removal and disposal of the contaminated product and legal fees, can also cause severe losses to a business's reputation and profits. A product recall of Tylenol in January 2010 resulted in a 31% decrease in Johnson and Johnson sales of all other non-prescription products across a nine-month period due to consumer mistrust (Zacs Investment Research, 2010).

There is a concerning trend with increased contamination events and product recalls. The Food and Drug Administration (FDA) conducted analysis of 642 microbiologically-related recalls between the years 2004-2011 and found a year-on year increase of the number of recalled products (Sutton and Luis, 2012). Indeed following a spate of non-sterile product recalls in 2017, the FDA released warnings to drug manufacturers of the contamination risks of Bcc in non-sterile water based drug products (FDA, 2017).

1.1.3 Existing Rapid Methods

There is an urgent need in the pharmaceutical industry to develop rapid and reliable QC microbiology procedures. Microbiological absence/presence tests are routinely performed using RMM in the healthcare and food production industries. Despite this, traditional culture-based methods are still widely used to assess microbiological quality of personal care and home care products. In 2010, a survey found that of 16 major manufacturers in North America and Europe, only three routinely used RMM (Rapid Test Methods Ltd, 2014).

At present, RMM in QC microbiology are only routinely used for species identification once traditional enumeration methods have been used to isolate colonies. Commercial automated platforms currently marketed to pharmaceutical industries include Applied Biosystems MicroSEQ systems, DuPont Riboprinter and Celsis ReACT. Applied Biosystems MicroSEQ systems and DuPont Riboprinter platforms follow the same workflow: a colony is picked from a clean culture and the DNA is extracted. Then the 16S ribosomal RNA (rRNA) gene is then amplified using universal primers in a thermocycling Polymerase Chain Reaction (PCR) instrument. Identification methods for the platform varies with the MicroSEQ systems reading PCR products in a capillary sequencer and then raw nucleic acid sequences are computationally compared against reference databases (Janda and Abbott, 2007). Bacterial identification in the DuPont Riboprinter system separates PCR products by electrophoresis and membrane profiles are compared to profiles of known organisms to provide species level identification (DuPont, 2010). The Celsis ReACT system however differs from both of these platforms as the Celsis RapiScreen platform is first used to amplify the present bacteria. The Celsis ReACT workflow then extracts bacterial DNA. 16s specific probes for various pre-

determined organisms are then introduced and hybridize to the corresponding DNA sequence. Probes are immobilized to the bottom of the tube by streptavidin and then a Celsis LuminACT™ substrate is added which causes the probes to emit light, confirming the presence of a specific organism (Charles River Laboratories Inc, 2016). An alternative method to the use of 16s identification is the parallel use of multiple bacterial 'group' specific primers, as employed by The Ibis T5000 universal biosensor instrument. This instrument aliquots extracted DNA into a 96 well microtiter plate, with each well containing various "group specific" primers for subsequent PCR amplification. PCR products are then analysed by mass spectrometry, where base composition of each sequencing product can be determined and referenced against a database to provide sub-species level organism detection (Ecker *et al*., 2008).

Whilst these commercial identification platforms aimed at pharmaceuticals offer rapid results, they are not able to do so without viable cellular material. At present there is no methodology or commercial platform able to apply rapid molecular methods for the quantitative identification of viable organisms.

1.1.4 Publications on Molecular Based Detection in Personal Care Products

To date, there have been only 12 publications on PCR based detection of bacterial contamination in pharmaceutical non–sterile products (Ragheb *et al.*, 2012; Maiuta and Schwarzentruber, 2011; Farajnia *et al.*, 2009; Karanam *et al.*, 2008; Samadi *et al.*, 2007; Škof *et al.*, 2004; Jimenez, 2001; Jimenez and Smalls, 2000; Jimenez *et al.*, 1998, 1999, 2000; Merker *et al.*, 2000). Of these, only 2 have cited a non-culture-based detection methodology (Farajnia *et al.*, 2009; Samadi *et al.*, 2007). This is because microbial contamination in pharmaceutical products is low, the product environment is hostile for organism growth and viability, and microorganisms can also be damaged or stressed during production processes, making recovery without enrichment difficult. The two papers, which experiment with a culture free method, then go on to use culture techniques to improve bacterial detection sensitivity from 10^4 CFU/ml of product to 10^2 CFU/ml and <10 CFU/ml. However, performing enrichments is time consuming, and these studies have taken an average of 25-27 h to obtain a result (Ragheb *et al.*, 2012; Maiuta and Schwarzentruber, 2011; Farajnia *et al.*, 2009; Karanam *et al.*, 2008; Samadi *et al.*,

2007; Škof *et al.*, 2004; Jimenez, 2001; Jimenez and Smalls, 2000; Jimenez *et al.*, 1998, 1999, 2000; Merker *et al.*, 2000). Of the two studies that have used a non-culture-based detection method (Farajnia *et al.*, 2009; Samadi *et al.*, 2007), only one completed this within the '7 hour shift' timeframe (Samadi *et al.*, 2007). However, this paper used a lysate treatment and boil prep DNA extraction method on a topical lotion artificially contaminated with Staphylococcus aureus. No description of the product is given, however topical lotions are far simpler chemical formulations than shampoos, being mainly water based and free of detergents. Furthermore, use of *S. aureus*, a hardy gram-positive bacterium, would to a significant extent be more protected from the toxic lotion environment than the gram-negative *Burkholderia* (Samadi *et al.*, 2007).

The majority of studies (11/12) use one or more of the specified 6 aerobic organisms found in the Absence of Specified Micro-organisms test as their model organism. However, a report by FDA cited *B. cepacia* as the most frequent cause of contamination (Jimenez, 2007). Only one paper on the development of methods for determining contamination of a personal care product used *B. cepacia* as a test organism has been published (Jimenez and Smalls, 2000).This is perhaps due to the difficulty in maintaining and storing Bcc organisms. Isolation and culture of Bcc strains is very problematic due to their long growth periods, differential susceptibility to stress tolerance, differential preference for carbon sources and poor passage viability (Vermis *et al*., 2004).

1.2 *Burkholderia Cepacia* Complex: a key contaminant in the personal care industry

Members of the Bcc are highly diverse and incredibly versatile and have been isolated from an array of complex and toxic consumer products such as antibacterial solutions, preserved pharmaceuticals and personal care products, and even petroleum goods (White *et al.*, 2011; Jimenez, 2007; Oie and Kamiya, 1996).

A testament to their catabolic ability, Bcc strains have the ability to degrade extremely hazardous and toxic products, such as ethers, toluene, phenols, and

trichloroethylene (present in petroleum products), polycyclic aromatic hydrocarbons (PAH's) (present in crude oils and coal) and 2,4,5–trichlorophenoxyacetic acid the principal ingredient of Agent Orange (present in herbicides) (Torbeck *et al.*, 2011). Commonly, Bcc strains have been isolated as fuel-contaminants, which complicates safe fuel storage, containment and remediation as well as its use in engine systems (White *et al.*, 2011).

Outbreaks of nosocomial *Burkholderia* infection have been caused by contaminated medical devices (Rutala *et al*., 1988), parenteral medicinal products (Zorrilla-Vaca *et al*., 2016; Singhal *et al*., 2015; Smet *et al*., 2015; Moehring *et al*., 2014; Dias *et al*., 2013; Yang *et al*., 2008; Fernández *et al*., 1996), nebuliser solutions (Estivariz *et al*., 2006; Ghazal *et al*., 2006; Balkhy *et al*., 2005), nasal sprays and solutions (Dolan *et al*., 2011; Cunha *et al*., 2007; Estivariz *et al*., 2006), oral laxative medication (Akinboyo *et al*., 2018; Marquez *et al*., 2017), indigo carmine dye (Gravel *et al*., 2002), ultrasound gel (Shaban *et al*., 2017; Nannini *et al*., 2015; Jacobson *et al*., 2006; Hutchinson *et al*., 2004), lubricants for catheterization (Ángeles-Garay *et al*., 2012), and commercially available gloves (Sommerstein *et al*., 2017). Most concerning is Bcc ability to survive and proliferate in commonly used anti-infective solutions and wipes which has been directly attributed to outbreaks of Bcc nosocomial infections (Romero-Gómez *et al*., 2015; Lee *et al*., 2013; Heo *et al*., 2008; Cascio *et al*., 2006). Contamination of used anti-infective solutions and wipes by Bcc bacteria can occur intrinsically (during manufacture) or with failure to use as directed in-use procedures such as dilution with non-sterile water or dilution in non-sterile containers. Furthermore, contaminated personal care products have also been implicated in nosocomial incidence of Bcc infection. Intrinsically contaminated alcohol-free mouthwash is particularly associated amongst patients who have respiratory vulnerability (i.e. intubation) (Martin *et al*., 2012; Kutty *et al*., 2007; Molina-Cabrillana *et al*., 2006; Matrician *et al*., 2000; CDC, 1998). In 2005, a Bcc outbreak originating from intrinsically contaminated alcohol-free mouthwash affected 9 states across the USA prompting investigations from infection control units in the hospitals, state health departments, and the Centers for Disease Control and Prevention (CDC) (Kutty *et al*., 2007). In total 22 hospitals were affected for 146 days, with new diagnosis stopping 5 days after the alcohol-free mouthwash were voluntarily recalled by the manufacturer. During this period Bcc colonized 105

patients with 48 suffering from active infection and is attributed to in 2 deaths from pulmonary infection. An array of molecular identification methods was used by the Research Laboratory and Repository at the University of Michigan and FDA including 16S ribosomal RNA (rRNA), recA polymerase chain reaction (PCR) with restriction fragment length polymorphism (RFLP) analysis, repetitive extragenic palindromic-PCR genotyping and pulsed-field gel electrophoresis (PFGE). *B. cenocepacia* was identified as the predominant contaminating strain (81 of 103 strains tested). Preservative efficacy studies on in use mouthwash and hermetically sealed units found inadequate levels of the preservative agent cetylpyridinium chloride. Although the manufacturer report was not available it appears contamination occurred at some point during the manufacturing process (Kutty *et al*., 2007).

In 2008, a contaminated moisturising body-milk affected 5 patients across 18-day period in a Barcelona teaching hospital intensive care unit (ICU) (Álvarez-Lerma *et al*., 2008). Patient outcomes were reported as bacteraemia (3 cases), lower respiratory tract infection (1 patient) and urinary tract infection (1 patient). *B. cepacia* isolates recovered from patients were analysed with PFGE analysis along with isolates recovered from hermetically sealed units of the moisturiser which confirmed they shared the same *B. cepacia* clone and the intrinsically contaminated moisturising body milk was the source of the outbreak (Álvarez-Lerma *et al*., 2008).

An outbreak of multi-species Bcc contamination of a moisturising cream resulted in *Burkholderia* bacteraemia in four ICU patients. Strain typing matched patient strains to strains in hermetically sealed moisturising creams. Once reported to the manufacturer, issues with the water filtration system were identified as the source of production contamination (Wiener-Well *et al*., 2014). There first reported outbreak from prefabricated moist washcloths occurred in two separate campuses of a north Germany tertiary care teaching hospital between June and October 2008. This extensive *B. contaminans* outbreak, colonised 61 ICU patients and resulted in severe infection of 14 patients (Martin *et al*., 2011).

In the face of the numerous publications citing *Burkholderia* as a cause of outbreaks amongst vulnerable hospitalized patients, there have been calls to include it on the

The Absence of Specified Micro-organisms test (Torbeck *et al.*, 2011). At present they are only considered an objectionable organism for certain pharmaceutical non-sterile products under "The Microbiological Attributes of Non-Sterile Pharmaceutical Products" and there is currently no microbiological method outlined in regulatory guidelines which is able to identify Bcc strains (Sutton and Luis, 2012).

Relatively little is known about the dissemination of *Burkholderia* species as process contaminants, due to a lack of detailed contamination reporting by manufacturers, where the product has exceeded the "Total Viable Count" species are commonly listed as "unidentified bacteria", and where products have failed on "Objectional Organisms" the identification methods are employed are usually biochemical and do not have the resolution to speciate. One of the only existing reports to review the microbiological causes of product recall was conducted in 2006, and analysed data reported by the FDA between 1998 to 2006. This found a that members of the Bcc and particularly *B. cepacia* are the most frequently recovered organisms. *B. cepacia* was present in 22% of non-sterile and 2.5% of sterile product recalls and contamination events (Jimenez, 2007). Another review of FDA product recalls between 2004 and 2011 found that for "Objectionable Organisms" in non-sterile products *B. cepacia* was again listed as the most common, accounting for 34% (Fig 1) (Sutton and Luis, 2012).

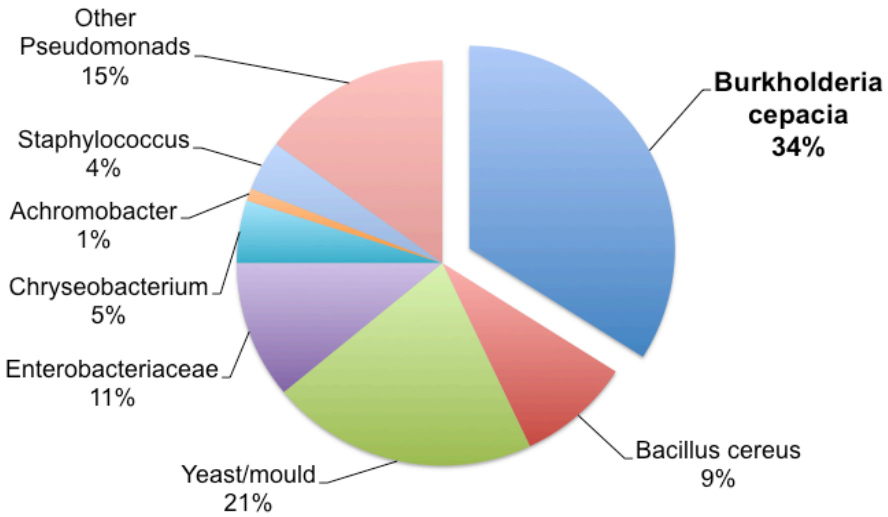


Figure 1. FDA data on USA product recalls for non-sterile products by objectionable organism between 2004 – 2011. Data taken from Sutton and Jimenez 2012.

A survey of 67 Bcc industrial process contaminants utilising MLST typing identifying strategies found that all formally named Bcc species along with unidentified novel species inhabit the environmental niche of the manufacturing facility, however of these *B. lata* and *B. cenocepacia* were the most common isolates, accounting for 25% and 18% respectively (Thomas, 2011).

1.2.1 Identification and Taxonomy

As with the evolving landscape of molecular strain typing, bacterial taxonomic identification and reclassification has advanced, evident from the classification history of members of the Bcc. *Burkholderia*, first described as *Pseudomonas cepacia*, was discovered in 1950 by German scientist W. H Burkholder, and later reclassified to *Burkholderia cepacia* in 1992 (Yabuuchi *et al*., 1992). *Burkholderia* is a rapidly growing genus, and from a mere seven species described in 1992, two decades of research has led to the formal identification of 25 species within Bcc (Table 1) and 74 *Burkholderia* species. Furthermore, this diverse genus is anticipated to keep growing, with recent analysis of 16srRNA sequences suggests there is a large number of candidate *Burkholderia* species awaiting formal description, along with the re-classification of existing poorly characterized species, and a large number of as of yet uncultivated species (Depoorter *et al*., 2016).

Table 1. Current List of the 25 *Burkholderia cepacia* complex isolates.

Species	Reference Paper
Burkholderia alpine	(Weber and King, 2017)
Burkholderia ambifaria	(Coenye *et al*., 2001)
Burkholderia anthina	(Vandamme *et al*., 2002)
Burkholderia arboris	(Vanlaere *et al*., 2008)
*Burkholderia catarinensis***	(Bach *et al*., 2017)
Burkholderia cenocepacia	(Vandamme *et al*., 2003)
Burkholderia cepacia	(Yabuuchi *et al*., 1992)
Burkholderia contaminans	(Vanlaere *et al*., 2009)
Burkholderia diffusa	(Vanlaere *et al*., 2008)
Burkholderia dolosa	(Vermis *et al*., 2004)
Burkholderia lata	(Vanlaere *et al*., 2009)
Burkholderia latens	(Vanlaere *et al*., 2008)
Burkholderia metallica	(Vanlaere *et al*., 2008)
Burkholderia multivorans	(Vandamme *et al*., 1997)
*Burkholderia paludis***	(Ong *et al*., 2016)
Burkholderia pseudomultivorans	(Peeters *et al*., 2013)
Burkholderia puraquae	(Martina *et al*., 2017)
Burkholderia pyrrocinia	(Storms *et al*., 2004)
Burkholderia seminalis	(Vanlaere *et al*., 2008)
Burkholderia stabilis	(Vandamme *et al*., 2000)
Burkholderia stagnalis	(Smet *et al*., 2015)
Burkholderia territorii	(Smet *et al*., 2015)
Burkholderia ubonensis	(Yabuuchi *et al*., 2000)
Burkholderia vietnamiensis	(Gillis *et al*., 1995)
*Pseudomonas mescoacidophilia***^	(Loveridge *et al*., 2017)

Footnotes:
*** This taxonomic name has been effectively published but not validly published under the rules of the International Code of Nomenclature of Bacteria (Bacteriological Code).*

^ Name awaits appropriate action by the research community to be transferred to another genus

Data taken from NCBI Taxonomy Browser, Available at: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=32008

Burkholderia are hard to identify using traditional biochemical and phenotypic traits. Traditional methods of molecular identification such as the use of selective media fail due to lack of specificity, as they also enrich closely related species such as *Ralstonia* (Krejčí and Kroppenstedt, 2006).

Various non-genomic tools have been developed for Bcc identification including whole cell protein analysis, SDS PAGE, whole fatty acid analysis, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) and protein arrays. However these tests were not sensitive enough to distinguish between the entire Bcc group (Suttisunhakul *et al*., 2017). This led to the development of PCR assays, with the first of its kind utilising amplification of the common 16S rRNA subunit and Restriction Fragment Length Polymorphism (RFLP). RFLP, the method of using restriction enzymes to cleave DNA at a specific site of varying size and then analysed using gel electrophoresis was a very common speciation technique in early molecular typing (Enayat, 2002; Tsipouras, 1987).

16s rRNA has been used for the past two decades for bacterial species level identification and diversity. 16s rRNA gene analysis pipelines involve the amplification of the entire 16s rRNA sequence by PCR primers. The nucleotide sequences of the amplicons are compared to other ribosomal sequence profiles using comprehensive prokaryotic databases such as the Ribosomal Database Project (RDP) (Cole *et al*., 2014). Sequence clustering at >99-97% homology indicates an existing species level identification, matches <97%-93% suggest a new species, and <93% suggest a new genus (Han, 2006). However, it was observed that the 16S rRNA genes between some closely related but distinct species were highly conserved, confounding accurate identification. One study observed that 16s rRNA gene sequencing at a 97% identity threshold underestimated diversity by 50% or more for the taxa *Pseudomonas, Burkholderia, Escherichia, Campylobacter* and *Citrobacter* (Rodriguez-R *et al*., 2018).

This then led to the development of a speciation method based on recA, a conserved protein located on the largest replicon of Bcc genomes (Mahenthiralingam *et al*., 2000). recA is present in a single copy and was shown to contain significant

variation to enable speciation. In the early 2000's recA PCR primer sequencing strategies successfully speciated four new Bcc strains (Payne *et al*., 2005).

Although this single gene technique was highly effective, a new approach was developed to align Bcc identification with the gold standard in bacterial identification, Multi Locus Sequence Typing (MLST) (Baldwin *et al*., 2005). MLST catalogues nucleotide polymorphisms in bacterial sequences for seven housekeeping genes and assigns them different allele types. The sum of the different allelic types at the seven loci then gives an allelic profile or strain type for each isolate. There is no ambiguity in the data as it is based on sequencing data, making it reliable and easily shared via databases across the scientific community. MLST has been used in the classification of novel species to the Bcc group (Vanlaere *et al*., 2008, 2009; Dalmastri *et al*., 2007).

At present debate surrounds the further subdivision of the *Burkholderia* genus into two major groups, which can be differentiated through their role as beneficial plant associated organisms or pathogenic to human, animal and plant hosts. However, this has been contested due to *Burkholderias* ability to ambiguously occupy multiple ecological niches and express pathogenicity dependent on environmental conditions (Coenye and Mahenthiralingam, 2014).

1.2.2 Pathogenicity

Bcc species are aerobic, gram-negative, motile non-fermenting bacteria and belong to the beta proteo-bacteria subgroup. These organisms are present in natural and man-made environments and exhibit exceptional metabolic diversity owing to their large genomes (Sousa *et al*., 2011). Bcc strains are very robust and can grow at temperatures ranging from 12 °C - 48 °C, survive on dry surfaces for a week and within water (including distilled water) for months (Vial *et al*., 2011). Bcc strains can also remain viable despite the presence of organic solvents, antimicrobial agents and product preservatives. For example, *B. cenocepacia* J2315 was seen to remain viable (with only a one log reduction in viable cells) after 60 minuets in the clinical hand disinfectant Hibiscrub^{TM}, containing the biocide chlorohexidine (4% w/v chlorohexidine gluconate) (Rose, 2009).

Members of Bcc are highly adaptable opportunistic pathogens and can infect plant, animal and human hosts. Historically, molecular biological and epidemiological studies have focused on the organism's ability to cause serious respiratory infections in cystic fibrosis (CF) patients (Drevinek and Mahenthiralingam, 2010). The effect of Bcc infection is dependent on strain type and host but generally results in adverse patient outcomes, prolonged in-patient stays and an increased probability of mortality when compared to other common CF pathogens such as *Pseudomonas aeruginosa*. Chronic infection is common amongst the CF community as Bcc species are highly resistant to antibiotics (Mahenthiralingam and Vandamme, 2005). Clinical studies of antibiotic therapy for Bcc isolates have found poor efficacy for antibiotics used alone, with resistance seen for every currently recommended therapy (Dalem *et al*., 2018; Shukla *et al*., 2018; Zhou *et al*., 2007; Aaron *et al*., 2000). Greater success of treatment is observed with double or triple antibiotic combinations. For example, one study found that *B. cenocepacia* K56–2 had a ceftazidime MIC of 128 μg/ml (16 times the CLSI breakpoint of 8 μg/ml) however when used in combination with 2 μg/ml moxifloxacin this was reduced to 2 μg/ml (El-Halfawy *et al*., 2017). Treatment of chronic Bcc infections with multiple antibiotic therapy is complicated by the emergence of multidrug resistance Bcc strains (Zhou *et al*., 2007; Saiman *et al*., 2003). All known Bcc strains have been associated with CF infections, however *B. cenocepacia* and *B. multivirons* are the most common, causing an estimated 85% of all Bcc reported infections. Although Bcc infections primarily affect immunocompromised patients, it is also able to cause infection in non-immunocompromised people (Gautam *et al*., 2011).

Pathogenic ability of Bcc strains varies greatly, and much research has been conducted into understanding virulence factors that attribute to life threatening infections (Leitão *et al*., 2017). Multiple virulence factors have been identified such as intrinsic resistance to antibiotics and disinfectants, a complex lipopolysaccharide structure and ability to form biofilm structures, highly developed quorum sensing pathways, and the presence of virulence associated structures such as genomic islands, cable pilus and flagella (Mahenthiralingam *et al*., 2005; Govan and Deretic, 1996).

Burkholderias plant pathogenicity was quickly followed by discovery of plant symbiotic relationships, in the form of nitrogen fixation in the rhizosphere of plants. This beneficial relationship has been identified in rice, peas, tomatoes and maize. Other desirable agricultural uses of Bcc members include the ability to act as a bio-pesticide, and in bioremediation of chemical breakdown such as trichloroethylene (O'Sullivan and Mahenthiralingam, 2005).

1.2.3 Genomic Analysis

The postgenomic era has enabled great advancement in *Burkholderia* taxonomy but also the relatively unknown mechanisms of molecular function. The first sequenced Bcc genome was of *B. cenocepacia* J2315, a globally disseminated cystic fibrosis (CF) infective isolate and was conducted by the Wellcome Trust Sanger Institute in 2000. The final published 8.06 Mb genome was available in 2009, taking nine years (Coenye and Mahenthiralingam, 2014). At present there are 123 complete genomes and 1062 draft genomes on the *Burkholderia* Genome Database (Winsor *et al*., 2008).

Bcc genomes are large and range in size from 7 to 9 Mb with a G+C base composition of approximately 67%. They consist of three major replicons, chromosomes 1 and 2 and what was previously considered chromosome 3 which is in fact a megaplasmid. Chromosomes 1 and 2, range in size from 3.3 to 3.9 Mb and 2.4 to 3.6 Mb, respectively with the megaplasmid ranging from 0.5 to 1.4 Mb in sequenced Bcc strains (Agnoli *et al*., 2014).

1.3 The Use of Preservatives to Protect Against Contamination

Antimicrobial agents cover a wide spectrum of compounds used to eradicate or inhibit micro-organisms. Biocides is a broad term for environmental antimicrobial agents often defined into their specific sub-classification: disinfectants, pesticides, antiseptics and preservatives (SCENIHR, 2009). Primitive chemical preservatives such as salt and sugar were used in early civilisation to prevent food spoilage. In current industrial manufacture, preservatives are incorporated into raw materials and finished products to prevent microbial contamination. Any formulation containing

water is vulnerable to microbial colonisation, therefore necessitating the need for antimicrobial preservatives to maintain product stability and composition (Rossmoore, 1995). Preservative agents are designed to protect product contamination that may occur during any stage of the product lifecycle, from manufacture, storage and use. As well as posing a threat to human health, product contamination causes organoleptic alterations, foul odour, changes in consistency and colour (Polati *et al*., 2007)

Due to their widespread use in consumer products, preservatives play a crucial role in daily life. Despite this, scientific research on biocides is relatively limited with research focused mainly on nosocomial antimicrobial use for human treatment of bacterial infections (Orth *et al*., 2006).

Preservative selection and dose must be carefully measured as there are associated disadvantages with toxicity and significant impacts on human health (Darbre *et al*., 2004). Along with in-use regulations of preservatives which differ in the country of manufacture and retail there are various manufacturing considerations on the selection and efficacy of an adequate preservative including:

1) The chemical properties of the preservative and maintaining the efficacy of both preservative and product on combination
2) The manufacturing process and packaging interactions
3) The intended use and environment of the finished product (Orth *et al.,* 2006).

Due to the multiple factors concerning preservative use, it is estimated that only one third of approved preservatives are commonly used (Reisch, 2018).

1.3.1 Current Regulatory Guidelines

Preservative agents are called biocides/antimicrobials and are incorporated into raw materials or finished products at low levels. Biocides are toxic and therefore are rigorously safety tested for adverse effects before approval and stringent maximum permitted levels are regulated. For Europe these guidelines are listed in the European Economic Community (EEC) Cosmetics directive annex 76/768/EEC (www.ec.europa.eu). Japan's Ministry of Health and Welfare stringently controls biocides, with very few permitted, and those approved are subject to many restrictions. In the USA the FDA prohibits or restricts the use of preservatives while

the Cosmetic, Toiletry and Fragrance Association (CFTA) finance and perform impartial reviews and safety evaluations of preservatives (Orth *et al*., 2006).

The method of determining the minimum preservative agent required to successfully defend against microbial spoilage is called Preservative Efficacy Testing (PET). In this test, personal care products are challenged with environmental type strains of bacteria, yeast and moulds which could also cause human harm, commonly gram-positive cocci (*S. aureus* and/or *Bacillus* spp.), gram-negative fermenters (*E. coli*) and gram-negative non-fermenters (*P. aeruginosa* and *B. cepacia*) (The European Pharmacopoeia Commission 2016).

1.3.2 Synthetic Preservative Systems

Ensuring products are adequately preserved is increasingly difficult. Growing concerns about safety has led to restrictions on preservatives regulated for use in the personal care industry. In the past 5 years EU authorities have banned three common synthetic preservatives and have decreased in use concentrations for two others (Reisch, 2016). Pressure from safety advocacy groups has resulted in negative consumer perception of certain preservatives, leading to a shift towards less controversial ones. Companies cannot develop novel preservative strategies due to the high cost development, lack of safety information and extended timescales for proper regulatory approval (Reisch, 2018). Despite the limited availability of effective preservatives, the commercial demand for effective preservatives is growing, with the $1 billion-a-year global preservative market forecast to increase between 4-5% annually (Reisch, 2016).

1.3.2.1 Isothiazolones

Isothiazolones are used to manage biofouling in a variety of industrial systems such as conventional water-cooling towers and water systems, fuel storage tanks, and pulp and paper systems as well as in the preservation of consumer products such as paints, adhesives, household and personal care products (Orth *et al*., 2006).

Isothiazolones possess a broad biocidal efficacy against bacteria, algae and viruses. They are a broad range preservative, owing to its high-water solubility, broad pH range, non-toxic biodegradability and compatibility with most ingredients.
Due to its potential to cause allergenic and irritation effects the use of isothiazolinone biocides is restricted. Benzisothiazolinone (BIT) is not permitted for use in personal care products in Europe or Japan but is unrestricted in the US. Methylisothiazolone (MIT) and chloromethylisothiazolinone (CMIT) were widely used in the personal care and cosmetics market as a preservation system (Halla *et al*., 2018). However, its popularity resulted in a rapid increase of methylisothiazolinone induced contact allergy and dermatological sensitisation from 1.5% in 2005 to 5.7% in 2013 (Schwensen *et al*., 2015). This led to various reductions of in-use concentrations followed by a full EU ban for use in leave on products by the Scientific Committee on Consumer Safety, which advises EU on the safety of preservatives (Erickson, 2016).

The mechanism of action for isothiazolinone biocides is considered a "two-step process", initially involving the instantaneous inhibition of cellular reproduction and key metabolic processes eventually leading to cellular death. Inhibition of cellular reproductive growth is caused by interruption of respiration (oxygen consumption) and energy (ATP synthesis) pathways of the cell through enzymatic inhibition, such as inhibition of dehydrogenases (Williams, 2007).

The theorized cause of cell death is oxidative interaction of thiol-containing peptides such as glutathione which is found both free in the cytoplasm and membrane bound (Collier *et al*., 1990). This is demonstrated experimentally where biocidal activity has increased with the presence of thiol-based molecules (Williams, 2007). Furthermore, disruption of core cellular metabolic pathways results in the generation of free radicals which also results in cell death (Chapman, 2003).

1.3.2.3 Parabens

Parabens are esters of alkyl hydroxyl benzoate. Parabens differ in the length of the carbon side chain, methyl/ethyl (short chain) derivatives and propyl/butyl (long chain) paraben derivatives (Krowka *et al*., 2017).

The mode of action of parabens is broad and non-specific. Parabens affect various biochemical processes including amino acid synthesis, especially glutamic acid and aspartic acid, protein synthesis and folding, and biosynthesis of nucleic acids. Parabens also are seen to decrease cellular pH, disrupt proton motive force, increase cellular permeability and cause leaching of integral cellular components (Garner *et al*., 2014).

Parabens, especially of the long chain variety are seen to have weakly estrogenic effects. A controversial 2004 study linked cosmetic parabens to human breast tumours, leading to a public revolt over their use (Darbre *et al*., 2004). Several scientific studies have identified flaws in this study and confirmed their continued safety at low levels, however public opinion remains low (Cassiday, 2013).

1.3.2.4 Formaldehyde Donors

Formaldehyde donors are active against bacteria and fungi and are commonly used as preservative agents in shampoos, shower gels, and liquid soaps. Formaldehyde donor preservatives are formaldehyde containing compounds which decompose over time to slowly release formaldehyde. This enables efficient preservation across in-use time periods (Halla *et al*., 2018). The rate and efficiency of formaldehyde release is contingent on various environmental factors such as pH, temperature, product matrix and storage time (Lv *et al*., 2015).

Free formaldehyde reacts on side groups, such as amide, amino, thiol, hydroxyl and carboxyl leading to crosslinking of proteins and DNA. In terms of proteins this leads to aggregation in the cell, increasing cellular toxicity, inhibiting metabolism and reducing cell division (Paulus, 2012).

Although it can be used safely at low regulated levels, formaldehyde is a carcinogen and it is attributed to human nasopharyngeal carcinoma and leukaemia (with prolonged exposure at much higher levels found in cosmetics) (Swenberg *et al*., 2013). This has made it unpopular as a preservative agent. Bowing to consumer demands, Johnson & Johnson removed formaldehyde and paraben preservatives from all baby care products (Safety and Care Commitment, 2017).

1.3.3 Natural Preservative Systems

In light of the growing resistance to traditional biocides and preservatives and with a greater spotlight on health-conscious consumerism, the demand for natural preservatives or so called "eco-preservatives" has increased. However, at present no legal or industry accepted definition for natural preservative exists. Natural preservatives exist in various forms including phytochemicals and naturally distilled extracts. Nature-derived preservatives are structures based on natural compounds but are synthesised and modified for better efficacy. Nature identical preservatives are preservatives with identical structures to those of natural origin. Although nature identical preservatives are found naturally they are often chemically synthesised (Ibarra and Johnson, 2008). There is a lack of clear consensus for what qualifies a natural preservatives, with various consumer safety groups exacting their own certification standards. Controversially, parabens are also found in natural sources and could be considered nature identical preservatives, however concerns on toxicity and metabolism of synthetic parabens means they are widely disregarded as an eco-preservative (Cassiday, 2013). The US based OASIS and the EU based ECOCERT allow the use of phenoxyethanol as a natural preservative, despite concerns from some safety groups that its synthetic production introduces chemicals not found in nature (Flanagan, 2011). Generally, in personal care preservation, nature identical preservatives are advocated and commonly encompass organic classes such as acid, salts and alcohols.

Despite increasing demand, the use of nature identical preservatives remains' low. The biggest drawback to most antimicrobials of natural origin (with the exception of phenoxyethanol) is that they fail to demonstrate a broad spectrum of antimicrobial activity (Steinberg, 2012). Furthermore, antimicrobial efficacy of nature identical preservatives requires higher levels than synthetic counterparts making them less cost effective (Lundov *et al*., 2011). In some cases, nature identical preservatives require 10 times the amount of a synthetic preservative to confer antimicrobial efficacy. Furthermore, high concentrations increase sensitization and allergic risks, and pose an increased probability for preservative dilution and failure (Browne *et al*., 2012). However, a recent study comparing in-product efficacy of nature identical preservatives against type strains of bacteria (*S. aureus*, *P. aeruginosa*) and fungi (*C. albicans*, *A. brasiliensis*) found that natural-identical preservatives out performed a

synthetic preservative and exhibited broad spectrum antimicrobial efficacy at lower levels. The synthetic preservative methylparaben:propylparaben 7.3% (v/v) required concentrations of 0.4% to achieve inhibition of all organisms, whilst concentrations for nature identical preservatives were either much lower or within the same range: Benzoic acid (0.13%), salicylic acid (0.13%), dehydroacetic acid/benzyl alcohol (8:92 w/w) (0.2%) and phenoxyethanol (0.5%) (Glavač and Lunder, 2018). This demonstrates that greater investigation is needed on the microbial efficacy of nature identical preservatives against industrial contaminant isolates.

1.3.3.1 Organic Acids

The use of organic acids has increased in industry, for example in agricultural practice across the world (but mainly adopted in European countries), prophylactic antibiotic livestock additive has been replaced with organic acids such as butyric acid which has a low mammalian toxicity but offer effective protection against pathogens (Fernández-Rubio *et al*., 2009; Leeson *et al*., 2005).

Organic acid preservatives such as salicylic acid and benzoic acid are most effective against fungi and less active against bacteria. These compounds are less “broad spectrum” than their synthetic counterparts as they need to be used at pH less than 5 and the salts need to be added to water to convert into the free acid (preservative agent) form which is difficult as their water solubility is poor (Browne *et al*., 2012).

Sodium benzoate naturally occurs in cranberries, prunes, greengage plums, cinnamon, ripe cloves and apples, but especially in berries (Vandevijvere *et al*., 2009). Sodium benzoate can also arise from the hippuric acid which is naturally present in most milk products such as yoghurt and cheese (Sieber *et al*., 1995). Sodium benzoate/benzoic acid is commonly used as a preservative in food, beverages, toothpastes, mouthwashes, dentifrices, cosmetics, pharmaceuticals and as an anticorrosive in technical systems such as an additive to automotive engine antifreeze coolants (Wibbertmann *et al*., 2005).

The mode of antimicrobial action is by disruption of the proton motive force which occurs in the cell membrane, and is a part of chemiosmosis, whereby an electrochemical gradient is maintained by pumping protons (H+) across the

membrane separating charges. This disruption of membrane activity then inhibits active transport (Russell, 2003). Furthermore, the increase in membrane potential and altered permeability along with the weakly acidic nature of the biocidal environment is thought to cause the intracellular pH to drop to below 5, decreasing the anaerobic fermentation of glucose through phosphofructokinase and inhibiting cellular growth and survival (Pongsavee, 2015).

Salicylic acid is named after the tree Salix Negra or Black Willow tree whose dark brown bark contains the natural salicylate derivative Salicin (Noleto-Dias *et al*., 2018) . Salicylic acid has many medicinal uses including as a treatment for dermatological conditions (Arif, 2015). It is the main component of the anti-inflammatory drug acetylsalicylic acid commonly called Asprin, in the production of wintergreen oil (methyl salicylate) and as an antiseptic and antifungal by topical application in veterinary medicine. Industrial uses include as a preservative in food, cosmetics and pharmaceuticals and as a chemical raw material for the synthesis of dyes, pesticides and rubber (Chemical Book, 2017).

Much like benzoic acid, the bactericidal effect of salicylic acid is attributed to its acidic properties, which affect the chemical gradient of ions across the cellular membrane, increase cellular permeability and disrupt the proton motive force as well as decreasing in intracellular pH (Helal, 2015). Salicylate has been shown to alter energy metabolism in many organisms through decreasing glycolysis, tricarboxylic acid cycle (Krebs cycle), pentose phosphate pathway, and amino acid metabolism (Riordan *et al*., 2007). In eukaryotic organisms, salicylate has also been shown to cause accumulation of reactive oxygen species (ROS) in the mitochondria, however a recent study in *E. coli* biofilms has also suggested that salicylic acid interacts with proteins that play a role in reactive oxygen species accumulation in prokaryotes (Cattò *et al*., 2017).

1.3.3.2 Alcohols

Alcohols have a history of use in hospitals as antiseptics for hand hygiene and disinfection of skin for surgical site preparation and also for small scale environmental surface disinfection (Weber *et al*., 2007). They are often considered a "milder alternative" to traditional biocides, with evidence from longitudinal studies

that topically applied alcohols (ranging from 60-90% concentration) cause little irritation and are safer for use then other biocides such as chlorohexidine and triclosan which commonly cause contact dermatitis (Löffler and Kampf, 2008; Pittet *et al*., 2007; Winnefeld *et al*., 2000).

Phenoxyethanol occurs naturally in green tea and chicory. Phenoxyethanol is a broad-spectrum antimicrobial, effective against bacteria, viruses, and fungi (although ineffective on spores). Phenoxyethanol is an effective preservative of pharmaceuticals, cosmetics and lubricant products (Krowka *et al*., 2014). Phenoxyethanol has many uses including perfume fixative, as a preservative of animal tissues (in the place of toxic formaldehyde), as an anaesthetic in fish, an ingredient in insect repellent, as a solvent in the production of cellulose acetate, dyes inks, resins and in adhesives (Maillard, 2002). Many alcohols are licenced as preservation systems for personal care products, however due to its low sensitization potential, stable non-pH dependent activity, solubility and global approval phenoxyethanol is preferentially used. Recent data cites phenoxyethanol as the most common antimicrobial for cosmetic products in the US in 2016 (Krowka *et al*., 2017).

Benzyl alcohol is found naturally occurring in fruits, teas and in plants such as jasmine, hyacinth, and ylang-ylang. It is active against gram-positive bacteria strains, with some activity against gram-negative (Committee for Human Medicinal Products, 2017). It is most commonly used as a preservation agent in medicines but is also found in personal care and cosmetics preservation due to its additional qualities as favouring and fragrance enhancers. Although not as popular as phenoxyethanol demand for benzyl alcohol is growing 5% annually (Technavio, 2017).

The mechanism of action for alcohols is the broad interruption of enzymes through binding to their active sites, disruption of protein synthesis and denaturation (Halla *et al.,* 2018). The biological effect for alcohols is concentration specific. At low concentrations Phenoxyethanol is also seen to be an uncoupler of oxidative phosphorylation (which inhibits the coupling between the electron transport and phosphorylation reactions thus disrupting ATP synthesis), induces proton

translocation and disruption of the proton motif force (PMF). At high concentrations alcohols may cause gross generalized loss of membrane function and leakage of cellular components leading to cell death (Denyer, 1995).

1.4 Microbial Resistance to Preservatives

Resistance to antimicrobial agents is wide ranging across all forms of life, affecting bacteria, fungi, parasites, viruses, humans, animals, plants, fish, insects and more. The development of resistance to antimicrobial agents is a serious concern (Davies and Davies, 2010). Current estimates for the global mortality rates for antimicrobial resistance is estimated to be 700,000, and these are predicted to increase to 10 million by 2050 and cause a global financial cost of 100 trillion US dollars (ONeill, 2014).

Despite efforts to understand and stop the emergence and dissemination of resistance it continues to spread across the world. In the case of antibiotic resistance, there are four severity-based classifications of resistance:

1. Antimicrobial resistance (AMR): resistance to specific classes of antibiotics.
2. Multidrug resistance (MDR): resistance across three or more antimicrobial classes.
3. Extensively drug resistant (XDR): resistance to all but two or fewer classes of antibiotics.
4. Pan-drug resistance (PDR): resistance to all antimicrobial classes.

The generation of novel antibiotic therapies cannot compete with growing resistance and antibiotic research has stagnated, threatening to send modern medicine into a pre-antibiotic era (Basak *et al*., 2016).

In the area of antibiotic resistance, resistance is defined as the ability for a micro-organism to grow at high concentrations of antibiotic for long or short periods. Resistance is usually attributed to inherited/acquired resistance mechanisms. Minimum inhibitory concentrations (MIC's) calculate the inhibition of a micro-organism at the lowest concentration of antibiotic/antimicrobial and this is used to

gauge levels of susceptibility or resistance (Brauner *et al*., 2016). Some strains classified as resistant can exhibit a susceptible phenotype but at higher doses, whereas some strains will exhibit total insusceptibility regardless of dose. Assessment of resistance differs for clinical and academic applications. Clinical resistance is defined as the failure of an antibiotic treatment against a MIC called a clinical breakpoint, defined by governing bodies of that geographic location, for the UK this is the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2006). Microbiological resistance is defined as MIC changes comparative from a susceptible wild type and is commonly used in resistance research (Wales and Davies, 2015). Tolerance is defined as a micro-organisms ability to survive short periods of high doses of antimicrobial treatment without a change in MIC. Tolerance is commonly caused by a reduction in bacterial functions such as dormancy which can be the result of mutations or environmental factors (such as poor growth conditions) (Kester and Fortune, 2013; Tuomanen *et al*., 1986; Lederberg and Zinder, 1948). Tolerance is often used to denote the prolonged survival of a bacterial population, while persistence is used to describe tolerance of a small sub-population of bacterial cells (Levin-Reisman et al., 2019). As tolerance and persistence occur without changes to the MIC these phenomena are best calculated with the plotting of minimum duration for killing (MDK) graph, which depicts the duration at which most (usually 99%) of the bacterial population/sub-population are killed by an antibiotic (Fridman *et al*., 2014). MDK graphs of tolerant populations show a slower rate of killing when compared to susceptible cells, and persister populations present as bi-phasic, with a rapid decrease in the majority of the population followed by a 'levelling' by the persistent sub-population (Balaban *et al*., 2004). Whilst little research has been done on preservative and biocide resistance, many studies have adopted terminology and methodologies used in antibiotic MIC testing and microbiological resistance.

Within the manufacturing plant environment there is likely to be sub-inhibitory concentrations of preservatives arising from residues within the production line caused by ineffective cleaning. Within product sub-inhibitory preservative arise from preservative degradation which can be caused by chemical interactions with the product, and even bacterial metabolism (Orth *et al*., 2006; Orth and Lutes, 1985).

At present there are reports of bacterial resistance to several preservative compounds including: benzalkonium chloride, benzisothiazolinone, benzoic acid, chlorhexidine, chlorophenol, dibromodicyanobutane, dimethoxy dimethyl hydantoin, formaldehyde, glutaraldehyde, hydrogen peroxide, imidazolidyl urea, iodopropynyl butylcarbamate, mercuric salts, methylisothiazolone, chloro-methylisothiazolone, methyl paraben, phenoxyethanol, phenyethyl alcohol, phenylmercuric acetate, propyl paraben, quarternary ammonium compounds, sorbic acid and trifluoromethyl dichlorocarbanilide (English, 2016; Chapman *et al*., 1998). This trend has become more concerning in recent times with an increase in preservative use in the personal care industry but a decrease in novel preservative discovery.

There are three ways in which antimicrobial resistance occurs which will be reviewed in the following chapter:

1. Intrinsic resistance – Physiochemical properties of the organism that does not change in response to antibiotic exposure.
2. Acquired resistance – Acquisition of a novel trait which confers resistance, for example a genetic change in the antibiotic target or regulatory element (enzyme or efflux pump).
3. Adaptive resistance – Exposure to sub-inhibitory concentrations of antimicrobials results in expression of resistance mechanisms, although in some cases the phenotype is sustained after the antimicrobial removed.

1.4.1 Intrinsic Resistance

Intrinsic bacterial resistance concerns innate cellular properties which result in reduced biocide susceptibility. Intrinsic resistance is frequently seen in bacterial biocide resistance but is very rare in antibiotic resistance whereby acquired resistance is more common (Fajardo *et al*., 2008).

1.4.1.1 Cellular Permeability and Outer Membrane Properties

The gram-negative cell wall system is far more complex than gram-positive bacteria and confers greater resistance (Silhavy *et al*., 2010). The gram-negative cell wall system is comprised of two membranes: an outer and an inner membrane (Glauert and Thornley, 1969). The presence of this double membrane system allows greater

control over the uptake and expulsion of small molecules such as biocides, and the periplasmic space enables enzymatic interactions, allowing the inactivation or modification of these antimicrobial agents (Reygaert 2018; Blair *et al*., 2014; Kumar and Schweizer, 2005). Antimicrobials must then permeate the double membrane barriers and in a high volume in order to cause any biocidal effect in the cell.

The bilayer outer membrane is comprised of an outer layer of phospholipid and lipopolysaccharides (LPS) and an inner layer of phospholipids Nikaido and Vaara, 1985; Kamio and Nikaido, 1976; Bladen and Mergenhagen, 1964). The outer layer controls cellular permeability through imbedded proteinaceous transport components such as lipoproteins (for the movement of fats), porins (which form hydrophilic channels) and efflux pumps (Delcour, 2008; Nikaido, 2003). To a lesser extent the inner cytoplasmic layer also contains proteinaceous transport elements. Commonly biocides target the cytoplasmic membrane and/or internal components, and many studies have demonstrated that the interaction of LPS, porins or efflux pumps can circumvent the effect of antimicrobials (Maillard, 2018; Chapman, 2003).

LPS, also known as endotoxin, are negatively charged molecules which contributes greatly to the structure and security of the outer membrane (Raetz and Whitfield, 2002; Nikaido and Vaara, 1985). As a major component of the outer membrane and covering approximately 75% of its surface, LPS is the first defence of the bacterium against toxic threats in the environment such as detergents and antibiotics (Gronow and Brade, 2001).

Bcc have a unique LPS structure when compared to other gram-negative bacteria. In non-Bcc bacteria the LPS comprises of three structures: a hydrophobic Lipid A, a core polysaccharide linked to Lipid A by 3-deoxy-D-manno-2-octulosonate (KDO) and a hydrophilic outer O-antigen side chain with repeating units (Caroff and Karibian, 2003). In Bcc however there are additional phosphate linked arabinose residues 4 amino-4 deoxyarabinose (Ara4N), fewer KDO and phosphate molecules, causing the net neutralisation of the outer membranes negative charge. This reduction in negative charge then makes the membrane less permeable for cationic and polycationic molecules. These observed changes in LPS of Bcc bacteria are presumed to cause intrinsic resistance to cationic compounds such as Polymyxin B

(Cox and Wilkinson, 1991). Polymyxin B is an antibiotic commonly used to treat gram-negative infections, and its molecular mode of action is through binding to the LPS and displacement of Mg2+ and interfering with Mg2+ intra-LPS molecular links which in turn decreases the integrity of the outer membrane structure. However, its cationic properties and the reduced net negative charge of Bcc results in a reduced binding affinity. It is instead utilised in Bcc selective media to differentiate from other gram-negative pathogens (Bodewits, 2010).

1.4.1.2 Porins

Gram-negative porins are hydrophilic channels that play an integral role in transport molecule regulation (Cowan *et al*., 1992; Nakae, 1976). There are two types of porins, ones which have functions in the movement of specific substrates which could otherwise not enter the cell (e.g. ferric iron chelates, maltose, nucleosides and vitamin B12) and general small molecule channels which are non-specific (Nikaido, 2003).

Studies have shown that in some Bcc strains a decreased porin size and porin content conferred greater levels of antibiotic resistance. In one study a *B. cepacia* strain exhibited 10 times the resistance against the beta-lactam nitrocefin when compared to *E. coli* and this was proposed to be caused by reduced comparative porin size in this strain which physically limited the diffusion of the antibiotic molecule (Parr *et al*., 1987). Examination of two nitrocefin resistant Bcc strains cultured from CF patients and one beta-lactam resistant mutant of the control reference strain, found that these strains contained reduced porin content when compared to the control Bcc reference strain (Aronoff, 1988).

Various studies have shown different outer membrane protein (OMP) expression patterns between sensitive and resistant strains exposed to isothiazolinone class of biocides. In *P. aeruginosa* exposed to classes of Isothiazolones, increased resistance which was associated with a novel OMP (Brözel and Cloete, 1994), the loss of OprD porins (Chapman *et al*., 1998) and the presence of the outer membrane protein T-OMP (Malek *et al*., 2002).

B. cepacia isolates with increased resistance to benzisothiazolinone had a different outer membrane profile than those without resistance. However, the method of speciation is not detailed and since this study was performed before advances in DNA sequence based taxonomy this may be another *Burkholderia* strain type or *Pseudomonas* (Chapman *et al*., 1998).

Although there is a link between porins and bactericidal susceptibility, it is hypothesized that this is only a piece of the puzzle and resistance is a multifactorial effort. Other systems are likely to be of greater importance, with porins only assisting resistance (Chapman *et al*., 1998).

1.4.1.3 Efflux Pumps

While prevention of antimicrobial entry into the cell is the foremost way for a bacterium to avoid succumbing to a bactericide, these cannot help once antimicrobials have made their way into the cell. Efflux pumps expel toxic compounds of any nature, including antibiotics, biocides, dyes, detergents (Piddock, 2006). Efflux systems can be general or specific and active efflux systems are attributed to higher levels of antimicrobial resistance. It has been hypothesized that exposure to any antibiotic or biocidal product will then increase efflux against other agents also. In the background of rising antibiotic resistance this implicates biocidal products as accelerating antibiotic resistance through “co-resistance” (Wales and Davies, 2015).

There are five families of efflux proteins which interact with antimicrobials:

1. The ATP binding cassette (ABC) superfamily
2. The major facilitator superfamily (MFS)
3. The multidrug and toxic-compound extrusion (MATE) family
4. The drug/metabolite transporter (DMT) superfamily which contains the small multidrug resistance (SMR) family
5. The resistance-nodulation-cell division (RND) superfamily

Recent studies have described two novel drug transporter families: antimetabolite transporters (the AbgT family) (Delmar and Yu, 2016) and the proteobacterial antimicrobial compound efflux transporters (the PACE family) (Hassan *et al*., 2015).

Efflux systems act as secondary transport systems and use the electrochemical separation of charges and proton motive force energy to expel biocides with the exception of the ABC superfamily which use ATP hydrolysis to power efflux systems (Li and Nikaido, 2004). Of major interest is the RND superfamily, as these are found widely throughout the outer cellular membrane. Regulation of the expression of these systems occurs at transcriptional level and involves DNA-binding proteins acting as repressors or activators that sense the presence of various compounds (Li *et al*., 2016).

For gram-negative bacteria, efflux has been indicated as a resistance mechanism for antibiotics and preservative agents, including β-lactams, quinolones, tetracycline, chloramphenicol, trimethoprim, sulfamethoxazole, aminoglycosides, chlorhexidine and quaternary ammonium compounds (QAC's) (Poole, 2005).

Efflux genes are located on the bacterial replicons and also on plasmids. For example, QAC resistance in gram-negative bacteria is associated with the chromosomal operon *emr* and also plasmid located genes *qacE* and *qacEΔ1* (Paulsen *et al*., 1993; Lomovskaya and Lewis, 1992).

The efflux systems involved in Bcc resistance are MFS, MATE and RND, with the RND efflux family the principal mediators of resistance (including multi-resistance) (Piddock, 2006). Evidence suggests that RND transporters coordinate with other transporters, for example, in *P. aeruginosa*, the *tetA* efflux pump has been shown to co-operate with *MexAB-OprM* and enables higher tetracycline resistance in the presence of both efflux pumps (Lee *et al*., 2000).

Burns *et al.,* (1989) first described the contribution of efflux transporters in *Burkholderia* in chloramphenicol resistance. This phenotype was caused by an outer membrane protein homologous to the *P. aeruginosa Mex-AB-OprM* RND efflux system (Burns *et al*., 1996). The entire five component efflux gene cluster was

subsequently isolated and characterized and named "*ceo*" for "cepacia efflux operon" encoding *CeoAB-OpcM*. It showed the ability to actively efflux chloramphenicol and salicylate out of the cell, and mediated resistance to trimethoprim and ciprofloxacin antibiotics (Nair *et al*., 2004).

There are 16 hypothesised RND systems in the *B. cenocepacia* genome and 245 putative RND proteins across other *Burkholderia* species. However as the genomic body of information grows this is set to change and increase (Bazzini *et al*., 2011; Perrin *et al*., 2010).

1.4.1.4 Inactivation and Modification

Enzymatic modification and inactivation have been reported in certain classes of antibiotic such as aminoglycoside and β-lactams. This phenomenon is not associated with cross-resistance (Wright, 2005).

Inactivation occurs in the periplasmic space and occurs when organisms produce molecules to interact and inactivate antimicrobials. *Burkholderia* create β-lactamases that breakdown and inactivate sensitive penicillin's and cephalosporin's (Hwang and Kim, 2015; Papp-Wallace et al., 2013; Chantratita *et al*., 2011; Poirel *et al*., 2008; Tribuddharat *et al*., 2003; Cheung *et al*., 2002; Trépanier *et al*., 1997; Godfrey *et al*., 1991).

Modification of aminoglycosides occurs by interactions with phosphotransferases. Bcc exhibit high intrinsic resistance to aminoglycosides, and although experimentally aminoglycosides-inactivating enzymes have not been found, aminoglycoside o-phosphotransferase and o-adenyltransferase genes are present in *B. cenocepacia* genomic sequences (Holden *et al*., 2009). Furthermore, a study by Mingeot-Leclercq *et al*., (1999) determined the increased expression of BCAL1756 gene in *B. cenocepacia* (which contains nucleotidyltransferase and phosphotransferase motifs and could have aminoglycoside-inactivating properties) when in the presence of an aminoglycoside antibiotic.

Biocides at sub-inhibitory concentrations can be metabolized by bacteria, and there are examples in the personal care industry where contaminated personal care

products had been caused ‘in part’ by the bacterial degradation of preservatives such QACs, benzoic acid, parabens, phenols, chlorhexidine, phenylethanol and formaldehyde (Orth *et al*., 2006; Russell, 2004; Russell and Chopra, 1996).

Many biochemical pathways naturally produce formaldehyde as a toxic by-product and therefore many organisms are capable of formaldehyde decomposition into harmless compounds (Marx *et al*., 2004). Bacterial resistance to formaldehyde-releasing preservatives occurs through the action of formaldehyde dehydrogenase enzymes, shown experimentally with high levels of formaldehyde resistance associated with increased synthesis of dehydrogenase enzymes (Orth *et al*., 2006). Bcc bacterial hydrolysis of formaldehyde preservatives occurs by the two known formaldehyde dehydrogenase metabolic pathways present in the genome (Marx *et al*., 2004).

Various bacterial species including Bcc have been seen to utilize parabens (p-hydroxybenzoic acid) for growth (Amin *et al*., 2010; Hugo, 2001). In *B. cepacia* and *Enterbacter* spp. decreased susceptibility to parabens has been attributed by increased expression of esterase enzymes which hydrolyse esters in parabens (Valkova *et al*., 2002; Close and Neilsen, 1976).

1.4.1.5 Biofilm Formation

Biofilms are complex bacterial structures, comprised of one or multiple microorganisms, which are surrounded by protective layer of high-density extracellular polysaccharides (EPS) (Costerton et al., 1978, 1987). Biofilm development begins with the adherement of free-floating planktonic cells to a surface, these then proliferate into a microcolony, and mature into multi-layered clusters of cells protected by a bacterial secretion matrix. Biofilm formation encompass various temporal stages, and can vary from the basic attachment stage, to a complete mature biofilm structure (Caiazza and O’Toole, 2004; Sauer *et al*., 2002; Stoodley *et al*., 2002; Tolker-Nielsen *et al*., 2000).

Bacteria in biofilms are more resistant to antimicrobial preservatives, disinfectants, and antiseptics than their sessile cell counterparts (El-Azizi *et al*., 2016; Scher *et al*., 2005; Gilbert *et al*., 2002; Costerton *et al*., 1999). Sessile cells are physiologically

distinct from their planktonic counterparts and much of their resistance is gained from their structure (Donlan and Costerton, 2002; Costerton et al., 1995, 1999). Alike sessile cells, enzyme mediated resistance and efflux mechanisms also form the basis for resistance in biofilms (Acker and Coenye, 2016; Buroni *et al*., 2014; Zhang and Mah, 2008; Bagge *et al*., 2004; Walters *et al*., 2003; Anderl *et al*., 2000). Biofilm structures enable greater antimicrobial resistance through the presence of an exopolysaccharide (EPS) matrix which limits entry and enables antimicrobial neutralization or binding, an increase in VBNC cells within the biofilm, and the presence of sub-populations of persister cells (Nadell *et al*., 2015; Marques *et al*., 2014; Kaspy *et al*., 2013; Flemming *et al*., 2007; Jefferson *et al*., 2005; Fux *et al*., 2003; Walters *et al*., 2003). Biofilms are formed by bacteria in response to various potential threats in the environment such as physical injury, dehydration, ultraviolet (UV) exposure, salinity and antimicrobial agents (Carvalho, 2017; Hathroubi *et al*., 2017; Hou *et al*., 2017; Kim and Chong, 2017; Marsden *et al*., 2017; Hostacká *et al*., 2010; Karatan and Watnick, 2009; Harrison *et al*., 2007).

Biofilm formation is observed equally in natural and man-made settings and are believed to be the favoured growth condition of many bacterial species owing to their greater survival potential (Costerton *et al*., 1999). *Burkholderia* are biofilm producers, like many from the beta-proteobacteria group. Biofilm-mediated resistance has serious implications for human health (resulting in recurrent infection and colonisation of medical devices) along with industrial manufacturing procedures (causing biofouling, machine obstruction and corrosion, product contamination) (Morton *et al*., 1998).

Clinically, biofilms are associated with prolonged illness and with treatment failure, with one study estimating a 12-22% increase in mortality rate with cardiovascular catheter-related biofilm infections (Crnich and Maki, 2002). Biofilm associated *B. cepacia* were seen to be up to 15 times more resistant to two common agents used to treat CF infections when compared to planktonically growing cells (Desai *et al*., 1998). Biofilms are notoriously difficult to remove, in both clinical and industrial settings. Bcc biofilms are resistant to common hospital and domestic disinfectants such as chlorohexidine and triclosan (Rose, 2009).

In recent years bacterial biofilm research has focused on the regulatory processes involved in formation, development, dispersal and architecture. A signalling molecule called, cyclic diguanosine monophosphate (ci-di-GMP) is known as biofilm initiator (Simm *et al*., 2004). In gram-negative bacteria high intracellular levels of ci-di-GMP is correlated with sessility. Two-component regulatory systems (TCS) are important in bacterial signalling and have been implicated in the formation of biofilms. They regulate cellular responses by detecting environmental ques and transfer a phosphoryl group to the required response regulator. For example, resistance in *P. aeruginosa* is linked to two small regulatory RNA's which along with other biological processes, regulate EPS production and biofilm formation (Karatan and Watnick, 2009).

Biofilm production and regulation in several bacterial species is also mediated by cell density dependent quorum sensing. Quorum sensing (QS) is an inter cell communication system which mediates stimuli response co-ordination through small signalling molecules called autoinducers. Once a threshold of autoinducer concentration has been breached, this then triggers a QS circuit, most commonly the co-ordinated expression of genes. Several studies have shown QS involved in regulation of various aspects of biofilm formation, from initial production and maturation, to structure and circulation (Brackman *et al*., 2009, 2011; Karatan and Watnick, 2009; Wopperer *et al*., 2006).

In gram-negative bacteria the primary QS circuit is controlled by the Lux operon which involves LuxI synthase which produces autoinducer molecules called N-acyl homoserine lactones (AHLs), which when bound to the LuxR promoter protein stimulates *pLuxR* to initiate transcription. This system is highly conserved in gram-negative species, although some organisms do have variant homologues (Turner and Manefield, 2002; Fuqua *et al*., 1994).

In Bcc the QS circuit is a two-part system analogous to the well characterised Lux operon called the CepI/R system. This system comprised of a CepI synthase which produces a AHL based autoinducer, most commonly N-octanoylhomoserine lactone (C8-HSL) although N-hexanoylhomoserine lactone (C6-HSL) has also been reported, which in turn binds to its corresponding regulating protein (CepR)

triggering a transcription response (Lutter *et al*., 2001). Many *Burkholderia* strains only possess the well characterized CepI/R system, however there is evidence of additional LuxI/R homologs such as BviI/R in strains such as *B. vietnamiensis* (Eberl, 2006), CciI/R in *B. cenocepacia* ET12 strain (Malott *et al*., 2005), TofI/R in *B. glumae* (Kim and Surette, 2004) and PlaI/R in *B. plantarii* (Solis *et al*., 2006).

Burkholderia pseudomallei, B. thailandensis, and the Bcc species *B. ambifaria* have demonstrated the ability to produce 4-hydroxy-3- methyl -2-alkylquinolines (HMAQs), a methylated derivative of the signaling molecule 4-hydroxy-2-alkylquinolines (HAQs) which had up to that time only been identified in QS mediated virulence systems in *P. aeruginosa* (Diggle *et al*., 2006). These three *Burkholderia* species contain a homologue to the *pqs*ABCDE operon found in *P. aeruginosa* called *hmq*ABCDEFG, which has two additional down-stream genes (Vial *et al*., 2008). Investigation of the role of HMAQ's in *B. ambifaria* QS signaling circuits show it triggers the use of a second signaling system CepI2/CepR2 system which uses hydroxylated AHLs, N-3-hydroxy-decanoyl-homoserine lactone (3OHC10-HSL), N-(3-hydroxydodecanoyl)-l-homoserine lactone (3OHC12-HSL). Phenotypes for the use of this secondary system over the traditional CepI/R system could not be identified (Chapalain *et al*., 2017).

QS in itself can be considered an important factor for antimicrobial resistance and virulence, as it is seen to mediate the core process involved such as the development of mature stage biofilm, swarming motility, the production of chitinases and extracellular proteases, and the production of ornibactin (a group of transport siderophores), type II/ type III secretion systems and oxidative stress responses (Subsin *et al*., 2007).

Several experiments have demonstrated that QS systems are integral for virulence phenotypes in *Burkholderia* species (Malott *et al.,* 2009; Eberl, 2006; Riedel *et al.,* 2003; Huber *et al.,* 2001). The importance of QS systems to virulence is further confirmed through the presence of a predicted AHL synthase and response regulator gene (*ccil*) on the *B. cenocepacia* virulence island cci, which also contained several other virulence factors (Malott *et al*., 2005).

1.4.2 Acquired Resistance

Resistance can be acquired through one of two known methods, by the mutation of existing genes on the bacterial replicon, or through the horizontal transfer of mobile genetic elements e.g. plasmids (Sandegren and Andersson, 2009).

Triclosan resistance is seen to be increased through mutation of the biocide target. Triclosan is the only single acting biocide, as it targets specifically fatty acid biosynthesis by inhibiting the enzyme enoyl reductase (enoyl-acyl carrier protein reductase, *fabI*). However, one study found mutations within the *fabI* gene within triclosan resistant *E. coli* (Orth *et al*., 2006; Russell, 2004a; Russell and Chopra, 1996). Furthermore, investigation of *P. aeruginosa* strains which were resistant to triclosan found they also contain mutations in the *fabI* gene (Poole, 2004).

Acquired resistance through the acquisition of plasmids has serious clinical consequences (McDonnell and Russell, 1999). Plasmids are known to encode virulence traits such as resistance to common antibiotics like beta lactams, tetracyclines, macrolides and chloramphenicol (Russell, 1997) and biocides such as chlorhexidine, QACs, triclosan and formaldehyde (McDonnell and Russell, 1999).

In the case of QAC's, mobile genetic elements encoding genes for proton-dependent efflux systems are present in gram-positive and gram-negative bacteria. Whilst the proportional contribution to the reduction of QAC susceptibility by these efflux systems is thought to be overall small, they are still considered significant in that can spread rapidly through the bacterial population (Chapman, 2003).

Several plasmid encoded genes are also thought to be involved in formaldehyde resistance by interference of outer membrane envelope proteins and increasing the presence of formaldehyde dehydrogenase (Dorsey and Actis, 2004; Kaulfers and Brandt, 1987).

In 1988 it was discovered that a single methicillin-resistant *Staphylococcus aureus* (MRSA) plasmid, pSAJ1 encoded significant resistance to five aminoglycoside antibiotics (kanamycin, gentamicin, tobramycin, amikacin) and four antiseptics (benzalkonium chloride, acriflavine, ethidium chloride and chlorhexidine)

(Yamamoto *et al*., 1988). This resistance phenotype was expressed when the plasmid was transferred into an *E.coli* host. Another MRSA plasmid, pSK1 is also seen to encode *qac* export proteins which gives rise to resistance to multiple biocides, notably the QAC's (McDonnell and Russell, 1999).

1.4.3 Adaptive Resistance

Adaptive resistance can occur when an organism is 'trained' to grow in gradually increasing concentrations of the biocide. Opposed to intrinsic and acquired resistance, which is an irreversible change in phenotype (independent of antimicrobial and environmental stimuli), adaptive resistance is an immediate resistance response to a stress stimuli (Fernández *et al*., 2011). Until recently it was believed that adaptive resistance is transient, however recent evidence suggests susceptibility is not always restored after the stimuli is removed (Russell, 2001). Several studies have demonstrated increased stable resistance after the stimuli has been removed, although this varies by bacterial species, dose and type of antimicrobial, and length of antimicrobial exposure (Fernández *et al*., 2011; Braoudaki and Hilton, 2004; Mawer and Greenwood, 1978).

Adaptive evolution to antibiotics by progressive subculture in sub-inhibitory concentrations has been extensively studied across multiple bacterial species due to its clinical significance (Fernández *et al*., 2011). Adaptive resistance mechanisms are complex, and it is common that bacteria will elicit a multifactorial resistance employing multiple mechanisms in response to an antimicrobial. An investigation of global gene expression of *B. cenocepacia* J2315 (ET12) strain which adapted stable resistance to Amikacin, Meropenem and Trimethoprim-sulfamethoxazole antibiotics found these strains exhibited increased expression in efflux, antibiotic degradation/modification, membrane function, and regulation pathways (Sass *et al*., 2011).

Differential resistance mechanisms are employed for different families of antibiotics due to their different cellular targets, however a common strategy involves reducing the accumulation of the toxic compound through active efflux (Piddock, 2006). For example, in *P. aeruginosa*, exposure to sub-lethal concentrations of aminoglycosides and ciprofloxacin (belonging to the quinolone class of antibiotics) is seen to increase

efflux through *MexXY-OprM* pump and *MexAB* systems respectively (Morita *et al*., 2014). However, the entry of aminoglycosides into the cell is thought to be aided by aerobic respiratory pathway, therefore exposure to sub-lethal levels also trigger a switch to the anaerobic respiratory pathway, and for ciprofloxacin which acts on DNA gyrase, a SOS response is induced to repair DNA damage (Brazas and Hancock, 2005).

Adaptive resistance to biocides is well known in industry and has been reported as far back as 1887 (Russell, 2004a). Although little research has been conducted surrounding the mechanisms of biocide-induced adaptive resistance, like antibiotics they are theorised to be multifactorial and specific to the particular biocide (Pérez *et al*., 2012; Fernández *et al*., 2011). Biocides (except for Triclosan) act on various cellular components, and therefore adaptive resistance to biocides involves general resistance mechanisms such as outer membrane changes, degradative enzymes and induction of efflux systems (Denyer, 1995; Hugo, 1967).

Adaptive resistance to biocides does give rise to co-resistance to other antimicrobials. However, this varies across strains (Wales and Davies, 2015). While developing stable adaptive resistance to three common biocides, with *E. coli* strain *O157* and *Salmonella enterica,* a study found that while stable adaptive resistance was achieved by both species, *E. coli* strain *O157* adapted quickly (after only two exposures) and exhibited broad increase in resistance to various antibiotics and biocides, but *Salmonella enterica* co-resistance was sub-type and biocide specific (Braoudaki and Hilton, 2004).

1.5 Project Aims

The funding for this PhD studentship was provided by the Biotechnology and Biological Sciences Research Council (BBSRC) Industrial Collaborative Awards in Science and Engineering (CASE) and Unilever PLC, a global company who produces a variety of consumer goods. The research was conducted in association with the Preservation and Applied Microbiology team at Unilever's Home Care and Personal Care R&D campus located in Port Sunlight, UK. The broad aims of this project reflect Unilever's interest in detecting and mitigating the contamination of home and personal care products by Bcc strains.

The aims for the research project were as follows:

1. To investigate the applicability of live/dead staining techniques in the assessment of contamination viability.
2. To develop a broad range quantitative rapid molecular method to detect and identify low level bacterial contamination (less than or equal to 10 CFU/g) in a variety of non-sterile home and personal care consumer products.
3. To develop a rapid narrow range detection and identification protocol for suspected product contamination. To assess the efficacy of the protocol against real world contamination samples from across Unilever home and personal care division.
4. To determine the preservative susceptibility of a large collection of Bcc clinical and industrial contaminant strains against synthetic and natural preservative agents using minimum inhibitory concentration methodologies.
5. To investigate the effect of strain/species, preservative type and adaptive resistance method on the potential generation of resistant populations by exposure to increasing preservative concentrations.
6. To investigate the molecular basis for antimicrobial preservative resistance through whole genome sequencing, Single Nucleotide Polymorphism analysis and GO enrichment.

Chapter 2: Materials and methods

2.1 Chemicals

All the chemicals used in this study were sourced from Sigma-Aldrich (Dorset, UK) and Fisher Scientific, (Loughbrough, UK). The preservatives used in this study were sourced from suppliers as listed in Table 2. The chemical properties of all the preservatives used in this study are given in Table 3.

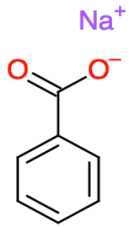
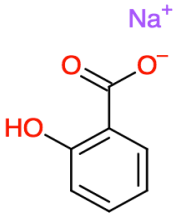
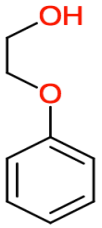
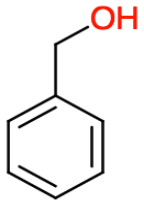
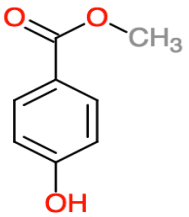
2.1.2 Preparation of Preservatives

Preservative stock solutions were prepared in sterile water or sterile dimethylsulfoxide (DMSO), and purity streaked for sterility. Final DMSO concentrations did not exceed 1% v/v. Stock solutions were made at a concentration of 10% v/v of active ingredient or at the maximum active ingredient concentrations achievable for preservatives with poor solubility. Stock solutions were stored at room temperature and never exceeded stated expiration dates. Preservative dilutions were prepared from stock solutions on the day of use for experiments**.**
Powdered acids sodium benzoate and sodium salicylate were diluted in water to give the active form, benzoic acid and salicylic acid, which they shall be referred to as from this point.

Table 2: A list of bactericides and their sources used in this study.

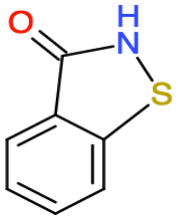
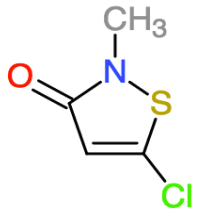
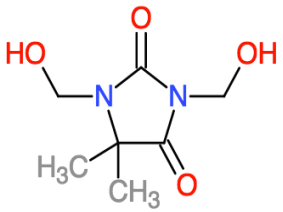
Brand	Chemical Name	Manufacturer	Location
Purox S	**Sodium Benzoate**	**Emerald Performance Materials**	**Rotterdam, Netherlands**
Salisod	**Sodium Salicylate**	**Salicylates & Chemicals PVT LTD**	**Hyderabad, India**
Phenoxetol	**Phenoxyethanol**	**Clariant**	**Frankfurt, Germany**
Proxel GXL	**Benzisolthiazolinone**	**Arch UK Biocides**	**Castleford, UK**
Purolan BA	**Benzyl Alcohol**	**Lanxess Distribution GmbH**	**Cologne, Germany**
Glydant LTD	**Dimethylol Dimethyl Hydantoin**	**Lonza**	**Basel, Switzerland**
Nipagin M	**Methyl Paraben**	**Clariant**	**Frankfurt, Germany**
Kathon CG	**Methylisothiazoline-chloromethylisothiazolinone**	**Rohm and Haas**	**Morges, Switzerland**

Table 3: Chemical properties of preservatives used in this study

Preservative Class	Acids		Alcohols		Parabens
Preservative Agent[1]	Sodium benzoate	Sodium Salicylate	Phenoxyethanol	Benzyl Alcohol	Methyl paraben
Chemical Formula	$C_7H_5O_2Na$	$C_7H_5NaO_3$	$C_8H_{10}O_2$	C_7H_8O	$C_8H_8O_3$
Chemical Structure					
Form	Powder	Powder	Liquid	Liquid	Powder
pH Range	5.0	5.0 – 6.0	3.0 - 8.5	3.5 – 8.0	3.0 - 11
Activity (%)[2]	100	100	100	3.5	100
Regulated Level (%)[3]	0.5	0.5	1	1	0.4

Footnotes:
1 International Nomenclature of Cosmetic Ingredients (INCI) designation
2 Active ingredient concentrations as listed on manufacturers 'Material Data Sheet'. See Table 2 for manufacturer information.
3 Regulated levels according to EU cosmetics directive 76/768/EEC, annex VI-list of preservatives for cosmetics.
**Manufacturers recommended level. U.S EPA Reregistration approval (Number 1258-1255) gives general recommendations of 0.02-0.35% for bacteriacidal action and use as household/laundry preservative at estimated concentrations of 0.05-0.15% of total weight of product.*

Table 3: Chemical properties of preservatives used in this study (continued)

Preservative Class	Isothiazolinones		Formaldehyde donor
Preservative Agent[1]	Benzisothiazolinone	Methylisothiazolinone-chloromethylisothiazolinone	Dimethylol Dimethyl Hydantoin
Chemical Formula	C_7H_5NOS	$C_8H_9ClN_2O_2S_2$	$C_7H_{12}N_2O_4$
Chemical Structure			
Form	Liquid	Liquid	Liquid
pH Range	4.0 - 12	6.0 – 8.0	7.5 - 8.5
Activity (%)[2]	20	1.5	55
Regulated Level (%)[3]	0.2*	0.0015	0.3

Footnotes:
1 International Nomenclature of Cosmetic Ingredients (INCI) designation
2 Active ingredient concentrations as listed on manufacturers Datasheets. See Table 2 for manufacturer information.
3 Regulated levels according to EU cosmetics directive 76/768/EEC, annex VI-list of preservatives for cosmetics.
**Manufacturers recommended level. U.S EPA Reregistration approval (Number 1258-1255) gives general recommendations of 0.02-0.35% for bactericidal action and use as household/laundry preservative at estimated concentrations of 0.05-0.15% of total weight of product.*

2.2 Media Preparation

All media were prepared using filtered water sterilised by autoclaving at 121°C. Lab M (Bury, UK) Luria Broth/Agar (LB/LA) and Tryptone Soy Broth/Agar (TSB/TSA) were used to grow and enumerate cultures for shampoo inoculation and recovery experiments and was made as per the manufacturer's instructions. A modified basal salt medium (BSM) was used for the Minimum Inhibitory Concentration (MIC) and bacterial selection experiments, due to its composition being closely related to nutrient restricted environment of a personal care product (Knapp *et al.*, 2013). The chemical composition of BSM is given in Table 4. Where preservatives were only active at pH 5, growth medium was buffered using citric acid (0.5 M) and sodium hydrogen phosphate Na_2HPO_4 (0.5 M) and an extra 5 g/L of Agar was added to solid media. Oxoid Burkholderia Cepacia Selective Media (Massachusetts, USA) was prepared per the manufacturer's instructions.

Table 4: Chemical composition of Modified basal salts medium (BSM) used in this study

Chemical	Molecular Formula	Grams per Litre (g/L)
Potassium Phosphate Dibasic Trihydrate	K_2HPO_4 $3H_2O$	4.25
Monosodium Dihydrogen Orthophosphate Monohydrate	NaH_2PO_4 H_2O	2
Ammonium Chloride	NH_4Cl	40
Magnesium Sulphate Heptahydrate	$MgSO_4$ $7H_2O$	0.2
Ferrous Sulphate Heptahydrate	$FeSO_4$ $7H_2O$	0.012
Manganese(II) Sulphate Monohydrate	$MnSO_4$ H_2O	0.003
Zinc Sulphate Heptahydrate	$ZnSO_4$ $7H_2O$	0.003
Cobalt(II) Sulphate Heptahydrate	$CoSO_4$ $7H_2O$	0.001
Casamino Acids	N/A	0.5
Yeast Extract	N/A	0.5
Glucose	C6H12O6	0.4% w/v

2.3 Bacterial Strains

Burkholderia clinical isolates were obtained from the University of Liverpool, Institute of Global Health and originated from Cystic Fibrosis (CF) patient sputum sample analysis. *Burkholderia* environmental strains were obtained from The University of Cardiff and were isolated as industrial process contaminants from factories and products of Unilever PLC, the CASE studentship sponsor.

Strain designations and properties for bacterial isolates are shown in Table 5. Species and strains were identified using selective media and *Burkholderia* specific primers, shown in Table 6.

Table 5: *Burkholderia* isolates from industrial and clinical sources identified by multi-locus sequence typing analysis

Group	Species	Strain Name	Isolation Source	MLST Sequence Type
Burkholderia Species	*B. gladioli*	BCC1317	Environmental industrial	0
		BCC1556	Environmental industrial	103
Bcc	*B. ambifaria*	BCC 284	Cystic fibrosis patient sputum, Liverpool UK	-*
Bcc	*B. arboris*	BCC1306	Environmental industrial	325
		BCC1307	Environmental industrial	325/327
		BCC1310	Environmental industrial	327/328
		BCC1312	Environmental industrial	328
		BCC1395	Environmental industrial	250/-*
Bcc	*B. cenocepacia*	J2315	Cystic fibrosis patient sputum, UK	-*
		BCC1283	Environmental industrial	322
		BCC1295	Environmental industrial	338
		BCC1558	Environmental industrial	324
		BCC1292	Environmental industrial	241
		BCC1316	Environmental industrial	316
		BCC1318	Environmental industrial	316
		BCC1320	Environmental industrial	340
		BCC1322	Environmental industrial	0
Bcc	*B. cepacia*	ATCC 25416	Cystic fibrosis patient sputum, Liverpool UK	0
		BCC1305	Environmental industrial	205
		BCC1404	Environmental industrial	3
		BCC1407	Environmental industrial	341
Bcc	*B. contaminans*	BCC1315	Environmental industrial	323
		BCC1323	Environmental industrial	0
Bcc	*B. dolosa*	BCC232	Cystic fibrosis patient sputum, Liverpool UK	0
Bcc	*B. lata*	BCC1406	Environmental industrial	119
		BCC1554	Environmental industrial	98
		BCC1287	Environmental industrial	98
		BCC1294	Environmental industrial	119
		BCC1296	Environmental industrial	119
		BCC1299	Environmental industrial	439
Bcc	*B. multivirons*	C5393	Cystic fibrosis patient sputum, Liverpool UK	0
		BCC1559	Environmental industrial	439
		BCC1560	Environmental industrial	337

Abbreviations:
Bcc : Burkholderia cepacia complex
Footnotes:
- Isolate identified to species level only, not assigned a strain type*

Table 5: *Burkholderia* isolates from industrial and clinical sources identified by multi-locus sequence typing analysis (Continued)

Group	Species	Strain Name or Other Strain Designations	Isolation Source	MLST Sequence Type
Bcc	*B. stabilis*	LMG14294	Cystic fibrosis patient sputum, Liverpool UK	0
		BCC1308	Environmental industrial	0
		BCC1325	Environmental industrial	0
		BCC1555	Environmental industrial	60
Bcc	*B. vietnamiensis*	PC279	Cystic fibrosis patient sputum, Liverpool UK	0
		BCC1301	Environmental industrial	0
		BCC1304	Environmental industrial	326
		BCC1309	Environmental industrial	333
Bcc	*Unknown BCC Species*	BCC1282	Environmental industrial	334
		BCC1300	Environmental industrial	333
		BCC1302	Environmental industrial	333
		BCC1303	Environmental industrial	335
		BCC1313	Environmental industrial	336
		BCC1314	Environmental industrial	0
		BCC1557	Environmental industrial	0
		BCC F642	Cystic fibrosis patient sputum, Liverpool UK	0
		BCC F655	Cystic fibrosis patient sputum, Liverpool UK	0
		BCC F651	Cystic fibrosis patient sputum, Liverpool UK	0
		BCC F768	Cystic fibrosis patient sputum, Liverpool UK	0

Abbreviations:
Bcc : Burkholderia cepacia complex
Footnotes:
- Isolate identified to species level only, not assigned a strain type*

Table 6: A list of primers used in this study

Primer Name	Nucleotide Sequence (5'-3')	Amplicon Size (bp)	Reference
27 fw	AGAGTTTGATCMTGGCTCAG	1465	*Lane, 1991*
1492 rv	TACCTTGTTACGACTT		
341 fw	CCTACGGGAGGCAGCAG	566	*Nadkarni et al., 2002*
907 rv	CCGTCAATTCMTTTGAGTTT		
BKH812 fw	CCCTAAACGATGTCAACTAGTTG	438	*Bergmark et al., 2012*
BKH1249 rv	ACCCTCTGTTCCGACCAT		
M13 fw	GTAAAACGACGGCCAG	1664*	*Invitrogen, 2013*
M13 rv	AACAGCTATGACCATG	199**	
Pse435 fw	ACTTTAAGTTGGGAGGAAGGG	251	*Bergmark et al., 2012*
Pse686 rv	ACACAGGAAATTCCACCACCC		

Footnotes:
fw: forward primer
rv: reverse primer
bp: base pair
**: with insert*
***: without insert*

2.4 General Methods

2.4.1 Growth Conditions

Bacterial cultures were revived from -80°C frozen stocks on agar growth media, at 30°C for environmental isolates and 37°C for clinical isolates for 18-19 hours, unless otherwise stated. Broth cultures were prepared by inoculating a sterile universal with 3-5 ml of growth media using the overnight revival plate and incubated on an orbital shaker at 150 rpm, at 30°C for environmental isolates and 37°C for clinical isolates for 18-19 hours.

2.4.2 Storage of Bacterial Isolates

Frozen -80°C stock collections were made from re-suspending fresh growth from a Tryptone Soy Agar plate into a 1.5 ml cryogenic tube (Nalgene New York, USA) containing, 1 ml of Tryptone Soy Broth and 8% (v/v) Dimethyl sulfoxide (DMSO).

2.4.3 Enumeration of Viable Bacteria

Total Viable Counts (TVC's) were performed by serially diluting cultures in buffered peptone solution and plating out 100 µl in duplicate onto Tryptone soy agar plates which were incubated at 30°C for 24-48 hours. The number of viable colonies were counted and expressed as colony forming units per ml (CFU/ml).

Chapter 3: Molecular Detection of Bcc Strains from Personal Care Products

3.1 Introduction

3.1.1 Microbiological Quality Control Testing

Pharmacopeia governs the physical and microbiological integrity of non-sterile pharmaceutical products such as toothpaste, mouthwash, shampoo, body wash, moisturizers and lotions. There are three pharmacopeias, European Pharmacopeia (EP), United States Pharmacopeia (USP) and Japanese Pharmacopeia (JP). In 1990, the Pharmacopeial Discussion Group formed to initiate international harmonization to avoid repeat quality control (QC) testing for products in different territories and streamline global trading. (Calam, 1995). The harmonization process was completed in 2009. Microbiological QC procedures cover three areas:

1. Total Viable Aerobic Count – A bioburden count for Total Aerobic Microbial Count (TAMC) and Total combined Yeasts and Moulds Count (TYMC) (European Pharmacopoeia, 2016a).

2. Tests for Specified Microorganism - A narrow range test to examine the product for the presence of up to 6 highly pathogenic organisms: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium, Salmonella abony*, *Candida albicans* and *Clostridium sporogenes* (European Pharmacopoeia, 2016b).

3. Microbiological Quality of Nonsterile Pharmaceutical Products - A recent extension to encourage the examination of products for specific microorganism's dependent on their composition and intended use (European Pharmacopoeia, 2016c). For example, alcohol-free mouthwash is known to be vulnerable to contamination of Bcc species, a prolific respiratory pathogen and therefore should be screened to confirm its absence before considered microbiologically safe (Torbeck *et al.,* 2011).

At present all three tests are still routinely performed using traditional testing methodologies such as plate count methods, selective media and biochemical tests, all of which rely on culture techniques (Jimenez *et al.,* 2000). Cultivation based QC methods are time consuming resulting in delays to the commercial supply chain such as prolonged product release, decreased time for completion of finished product and additional costs for stock storage (Sutton, 2006). These methods are also prone to error and consequently result in repeat QC testing and out-of-specification investigations and the release of contaminated products which can result in product recalls (Sutton and Cundell, 2004). Product recalls are costly not only in the direct costs associated with product removal but to a company's brand and reputation. In the food industry, the direct costs associated with product recalls are estimated to be 10 million dollars (Grocery Manufacturers Association, 2010).

Furthermore, current contamination detection methods employed in QC investigations for any suspected contamination event involve pre-enrichment, whereby contaminated products and/or the isolated contaminating organism is incubated at 30-37°C for 2-3 days in growth promotive media such as TSA (Jiang *et al.,* 1998; Olsen *et al.,* 1995). Pre-enrichment is used as contaminating bioburden is low and the toxic environment of the product and/or the production process can result in damaged or stressed cells making recovery without enrichment difficult (Rathnayaka, 2011; Piknová *et al.,* 2002; Moreno *et al.,* 2001). However, a rapid response is imperative for appropriate factory decontamination and recall procedures therefore the use of pre-enrichment is not efficient for the fast-paced modern production environment.

Recovery is often difficult even with enrichment methods as damage or stressed cells, when exposed to 'nutrient rich" agars then suffer from nutrient shock effects, causing cell death. Despite this, TSA, a nutrient rich medium, is recommended by current European Pharmacopeia for total viable counts of home and personal care products (European Pharmacopoeia, 2016a). However, less nutrient rich and potentially superior agars exist such as LB agar which is commonly used to cultivate the gram-negative *E. coli* and Reasoner's 2A (R2A) agar which is used in water testing (Reasoner and Geldreich, 1985; Liao *et al*., 2013). The addition of catalase to media is seen to increase recovery of damaged, stressed, VBNC and otherwise viable

cells (Anvarian *et al*., 2016; Bang *et al*., 2007; Mizunoe *et al*., 2000). Catalase is an antioxidant enzyme and it degrades hydrogen peroxide, which forms naturally in bacterial cells from aerobic respiration. Stressed cells are more sensitive to the toxic accumulation of hydrogen peroxide, which results in oxidative stress and cell damage/death (Leist *et al*., 1996).

3.1.2 *Burkholderia Cepacia* Complex as Industrial Contaminants

The "Microbiological Attributes of Non-Sterile Pharmaceutical Products" test encourages the manufacturer to independently consider the microbiological vulnerabilities of formulations. This arose from the rise of contamination events caused by previously unconsidered organisms such as the Bcc (Sutton and Luis, 2012). A recent review by the U.S food and drug administration (FDA) into product recalls over the last decade reported *B. cepacia* contamination in preservative treated non-sterile and sterile pharmaceutical products including eyewash, nasal spray and mouthwash (Torbeck *et al.,* 2011). This study is likely to have incorrectly identified strains as *B. cepacia* due to a lack of accurate taxonomic identification. Bcc species are genomovars, genetically distinct but phenotypically similar species, necessitating molecular and genomic tools to assist in the identification of these contaminating bacteria. Indeed, as these taxonomic methods have been created and applied and reapplied to existing strains, the Bcc species collection has grown and is currently comprised of at least 17 different genomovars and 74 *Burkholderia* species.

The *Burkholderia* genus stand out as being extraordinarily metabolically diverse and adaptable, capable of survival in extreme environmental niches owing to their stable complex biofilm structure, ability to utilise a range of substrates for growth and innate antimicrobial resistance (Coenye and Mahenthiralingam, 2014). *Burkholderia* species are able to enter a viable but non-culturable state in which they can evade detection by TVC and ATP bioluminescence methodologies (Barlasov *et al*., 2014). For example, a paraben preserved oral antihistamine which passed QC testing was discovered to be contaminated with *B. cepacia* six months after manufacturing. The contamination investigation found the total bioburden increasing from 10 CFU/ml to 1475 CFU/ml when repeat testing was conducted (Eissa, 2016).

Burkholderia species are associated with the contamination of preserved products, as well as bacterial infections in the CF community, leading to concerns that poorly preserved products may select for resistant strains which then circulate in the environment, and have a direct impact on human health (Barlasov *et al*., 2014).

In summary, members of the Bcc are uniquely adapted to avoid QC detection methods, resist chemical preservatives and proliferate in low nutrient conditions and have the pathogenic capacity to cause disease in non-immunocompromised consumers. It is for this reason that Bcc organisms were chosen as the model organism in this project on the development of universally applicable molecular-based detection protocols for non-sterile pharmaceutical products.

3.1.3 Molecular Methods of Detection

Advances in PCR and Next Generation Sequencing (NGS) technological capabilities have resulted in sensitive and fast methods of microbial detection even at very low limits (at less than 10 CFU) and reliable detection of non-viable and/or viable but non-culturable organisms (Karanam *et al*., 2008). Molecular methods of detection supersede standard bacteriology methods, as they are rapid, sensitive and highly accurate. The use of molecular methods for the detection of specific pathogenic bacteria in clinical samples, sterile pharmaceutical products and the food industry has been well documented (Rodríguez-Lázaro and Cook, 2013; Ragheb *et al*., 2012). However, the application of rapid molecular methods to analyze pharmaceutical non-sterile products has been limited due to accessibility of PCR and sequencing equipment and high costs (Mardis, 2011). However, in recent years, commercial PCR and sequencing costs have fallen dramatically making this technology affordable and accessible.

Home and personal care products cover a diverse range of different consumables, including cosmetics, shampoo, deodorants, household disinfectants and laundry detergents. These have highly variable chemical formulations and physical characteristics within this group may intervene in the production of a broad molecular biological testing method. For example, a shampoo would be more difficult to examine using molecular methods than a topical cream or lotion because it contains numerous foaming agents, surfactants, emulsifying agents, antimicrobial

agents and wax residues. Chemical substances such as ionic detergents and polysaccharides are commonly found in personal care products and are known to inhibit or affect PCR processes (Schrader *et al*., 2012). A 0.01% concentration the ionic detergent sodium dodecyl sulphate (SDS) will inhibit the activity of Taq DNA polymerase by 90%, and a 0.1% concentration will inhibit Taq activity by 99.9% (Konat *et al*., 1994). Often product composition is not reported in papers however the complexity of the sample matrix is integral to the demonstration of a robust method, a low complexity product is not equivalent to a high complexity product, making it difficult to cross compare reported sensitivity from studies assessing PCR based detection of bacterial contamination in pharmaceutical non –sterile products.

In order for a molecular method to be employed routinely in a QC microbiology lab, a simple and cost-effective protocol are essential. The methodology of boiling bacterial culture for preparation of DNA template to use directly in PCR (known as boil-prep) is well cited in the literature, as it is a rapid, cheap and technically easy procedure to obtain sufficient genomic DNA for PCR (Talkington, 2013).

In current molecular methods for detection, often the 16S ribosomal RNA (rRNA) gene is used as the target as it is ubiquitous in all bacterial species (Rajendhran and Gunasekaran, 2011). The 16S subunit has highly conserved regions which act as primer hybridization points and highly variable regions which can discriminate between taxa and in some cases provide species level identification (Janda and Abbott, 2007). rRNA sequence profiles are well characterized, and various reliable reference sources such as the Ribosomal Database Project (RDP) are available (Cole *et al.,* 2014).

RNA can be considered a superior target for detection than DNA due to its ability to indicate the presence of live cells and the greater abundance of ribosomal RNA present in the cell (Stewart *et al*., 2010). Indeed, 16S rRNA targeting methods (Fluorescence in situ hybridization (FISH) and quantitative probing methods), coupled with qRT-PCR have become the gold standard method for microbial detection and assessment of bacterial abundance in microbial ecology (Wagner and Haider, 2012; Kong *et al*., 2010; Cai *et al*., 2008). However, it is likely that when slow-growing or VBNC cells are present in a sample, the RNA content is very low,

making its cellular abundance equivalent to DNA (Willi *et al*., 2018; Svenningsen *et al*., 2017).

Dye based inhibition of DNA from non-viable cells during PCR amplification known as viability-PCR can provide an estimate of cell viability. The application of the viable PCR concept was first cited in 2003 using the DNA dye ethidium monoazide and is also known as EMA-PCR (Nogva *et al*., 2003). In EMA-PCR ethidium monoazide selectively enters cells with compromised cell membranes (i.e, non-viable cells). Exposure of these cells to light (550 nm), then causes photolysis of the azide into a highly reactive nitrene radical, which then covalently cross-links to cellular DNA, inhibiting disassociation of double stranded DNA (dsDNA) into single stranded DNA (ssDNA), thus preventing DNA amplification. Furthermore, unbound excess dye reacts with water to produce hydroxylamine, preventing unspecific uptake by viable cells (Elizaquível *et al*., 2014; Soejima *et al*., 2011). The distinction of viable bacterial cells from vegetative non-viable cells is of particular importance to food and water testing, and clinical microbiology. It is also of concern to pharmaceutical non-sterile products, where the pharmacopeial regulations are only concerned with viable cells capable of product spoilage or potential harm to consumers. Therefore, employment of EMA-PCR for the selective amplification of DNA from viable and potentially harmful contaminating organisms would be advantageous in nucleic acid based detection of contamination in pharmaceutical non-sterile samples.

3.2 Chapter Aims

The aims of this chapter were as follows:

1. To investigate the use of DNA or RNA as a target for the nucleic acid detection protocols.
2. To examine the use of viability staining techniques to provide an estimate of live and potentially pathogenic bacteria from cultures and artificially contaminated personal care products.
3. To investigate the optimal diluent and recovery medium for standard culture techniques to quantify bacterial contamination.
4. To examine the optimal methodology for the broad range detection of general bacteria in artificially contaminated personal care products using nucleic acid extraction, amplification and visualisation.
5. To explore the optimal methodology for narrow range detection and identification of *Burkholderia* or *Pseudomonas* species in artificially contaminated personal care products using neat lysates and nucleic acid amplification and visualisation.
6. To examine the utility of a narrow range detection protocol for the detection and identification of *Burkholderia* or *Pseudomonas* species using "real world" contaminated personal care products.

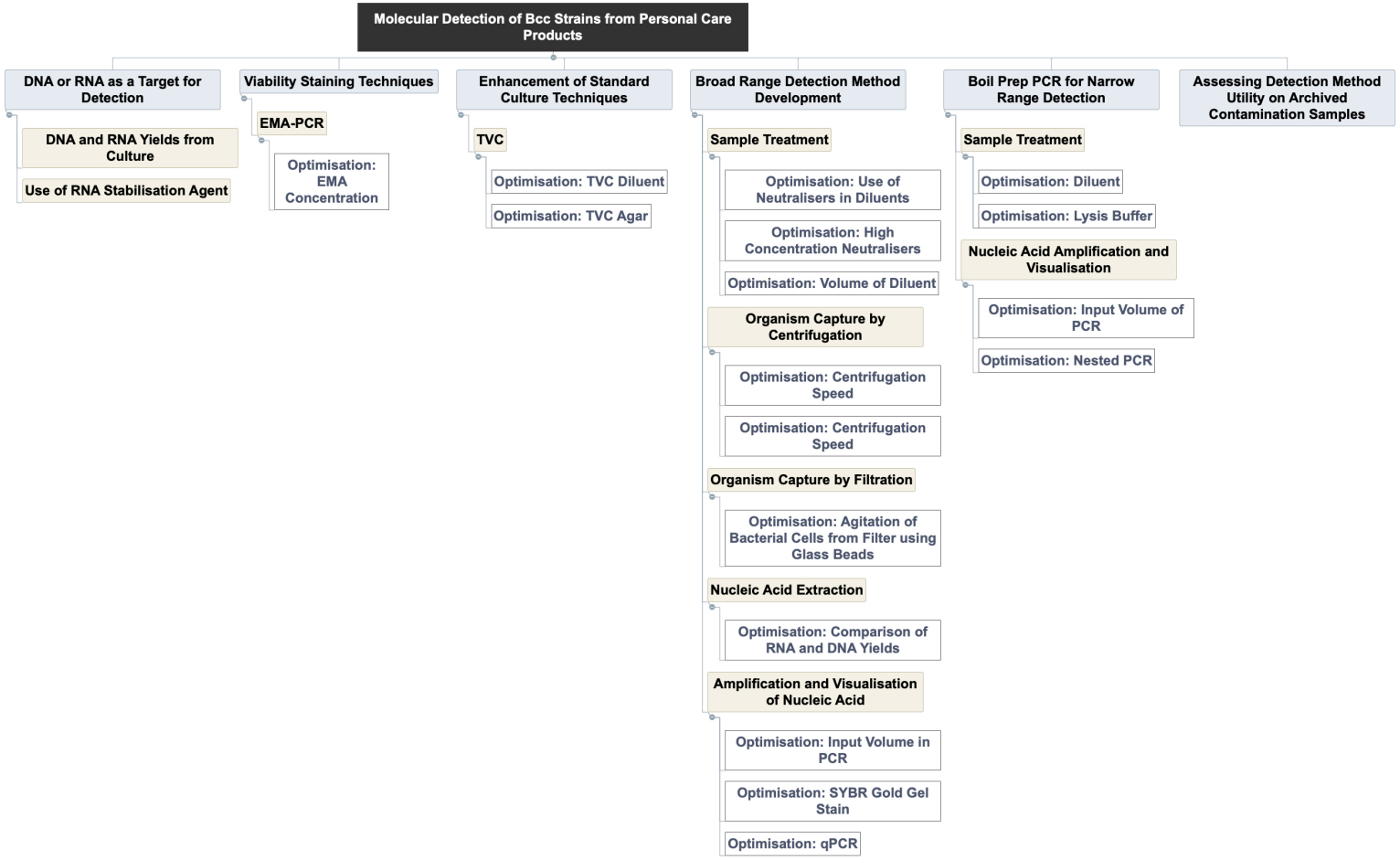


Figure 2: Hierarchy graph of chapter 3 aims (blue boxes), methodological approach (yellow boxes) and method optimisations (white boxes).

3.3 Methods

3.3.1 Method Development Using Bacterial Cultures

Prior to methodological development using artificially contaminated personal care products, small scale experiments were performed on bacterial cultures only to ascertain whether RNA protocols could be truncated to fit in the '7 hour shift', if DNA or RNA should be used for detection purposes and also if DNA binding viability dyes could be incorporated into the method (Figure 2).

3.3.1.1 Optimisation of RNA Protocols

RNA extractions were performed in duplicate to investigate the effect of shortening the incubation period with Qiagen's RNAlater solution (Limburg, Netherlands) from 24 h to 4 h. Four biological replicates were performed using Bcc clinical strains (*B. cepacia* ATTC25416*, B. vietnamesis* pc279*, B. multivirons* c5393 and *B. dolosa* bcc232) were grown in 5 ml LB broth at 37°C for 12 h and RNA extractions were performed using the Qiagen RNeasy mini-kit (Limburg, Netherlands) as per the manufacturer's instructions. The RNA yields were quantified using a Nanodrop Spectrophotometer (ThermoFisher, Massachusetts, USA). The average RNA yields of the four biological replicates and standard error were reported.

3.3.1.2 Comparison of DNA and RNA yields

To investigate DNA and RNA yields from cultures, RNA and DNA extraction were performed on two Bcc clinical strains (*B. cepacia* ATTC25416 and *B. cenocepacia* J2315) which were grown in LB broth at 37°C for 12 h. RNA and DNA extractions were performed in triplicate. The RNA extraction was performed using the Qiagen RNeasy mini-kit (Limburg, Netherlands) and the DNA extraction was performed using Bioline ISOLATE II Genomic DNA kit (London, UK), according to the manufacturer's instructions. The RNA and DNA yields were quantified using a Nanodrop Spectrophotometer (ThermoFisher, Massachusetts, USA). The average nucleic acid yields of the two biological replicates and six technical replicates with standard error were reported.

3.3.1.3.1 Viability PCR Methods - Ethidium Monoazide PCR (EMA-PCR)

The EMA methods were derived from published methodologies by Agustí *et al.,* 2010 and Delgado-Viscogliosi *et al.,* 2009. EMA (Molecular Probes Inc, ThermoFisher, Massachusetts, USA) was stored at -20 ºC in absence of light and prepared as a stock solution (5 mg/ml) in sterile ethanol as per the manufactures' instruction. Ethanol is toxic to bacterial cells, therefore 2% ethanol (equivalent to the maximum amount of EMA solution which would be used) was added to TSB and inoculated with overnight plate growth of *B. cenocepacia* strain 1558 and incubated at 30°C for 12 h. The optical density (OD) value was measured by spectrometer at 600 nm. A control of TSB without 2% ethanol was also performed using the same method. There was little difference observed in OD values for *B. cenocepacia* strain 1558 growth with or without 2% ethanol (data not shown).

To compare relative efficacy of EMA – PCR, experiments were performed on viable and non-viable cells. To prepare non-viable cells, a 2 ml culture *B. cenocepacia* strain 1558 cells were heated at 85 °C for 30 minutes in a heat block. Cell viability was checked by spreading 100 µl onto duplicate TSA plates and incubation at 30 °C for 12 hours. No viable cells were detected.

10 µl/ml EMA stock solution was added to opaque 2 ml microcentrifuge tubes containing 1 ml of overnight *B. cenocepacia* strain 1558 cell culture approximately ~1x10^9 CFU/ml. EMA treated samples were incubated in the dark room for 10 minutes. After incubation, tubes were exposed to 650 nm light for 15 minutes for photoactivation. Cells were centrifuged at 5000 × g for 10 minutes and the pellet was resuspended in 100 ml PBS for DNA extraction. DNA extraction was performed using Bioline ISOLATE II Genomic DNA Kit (London, United Kingdom) according to the manufacturer's instructions. DNA was serially diluted to 10^{-8} and PCR amplification was performed on each dilution by adding 2 µl of diluted DNA to 48 µl of PCR mixture including 25 µl Bioline BioMix™ Red (London, UK), 2 µl 16srRNA forward primer (See Table 6), 2 µl 16srRNA reverse primer 1492r (See Table 6) and 19 µl RNA free water. PCR products were visualised using a UV transilluminator, on a 1% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany), using electrophoresis techniques. PCR products were purified using Bioline ISOLATE II PCR and Gel Kit (London, UK)

according to the manufacturer's instruction. PCR products were quantified using a Nanodrop Spectrophotometer (ThermoFisher, Massachusetts, USA). The experiment was repeated in triplicate with *B. cenocepacia* strain 1558.

3.3.1.3.2 Optimisation of EMA protocols

In order to calculate the optimal EMA concentration viable and non-viable bacterial cultures were treated with 5 µl/ml and 7.5 µl/ml of EMA. EMA-PCR was performed as previously described with the exception that extracted DNA was not diluted for PCR amplification. The experiment was repeated with *B. cenocepacia* strain 1558 to give two biological replicates.

3.3.2 Method Development using Artificially Contaminated Personal Care Products

3.3.2.1 Enhancement of Total Viable Counts

Total viable counts were conducted as described in section 2.4.3 but with various media and diluents to investigate the optimal combination for bacterial cell recovery (Figure 2). To simulate a contaminated product, shampoo was inoculated in a 1/10 ratio with overnight of Bcc culture. A control of PBS was inoculated with overnight Bcc culture. The inoculated PBS control/shampoo sample was serially diluted in a diluent and left for 30 minutes to allow bacterial recovery.

3.3.2.1.1 TVC Medium Design

The serially diluted PBS control/shampoo sample was plated on 7 different media and supplement combinations in order to investigate nutrient stress as a parameter to consider in medium design; TSA, R2A, R2A + 0.01% Bovine Catalase, 50% R2A + 50% LB Agar, 50% R2A + 50% LB Agar + 0.01% Bovine Catalase, LB Agar, LB + 0.01% Bovine Catalase (Sigma Aldrich, Missouri USA). A mix of 50% R2A Agar and 50% LB Agar was made to create a high salt, partially nutritious medium which should promote growth within a shorter timeframe. 100 µl of 0.01% Catalase was spread on the plates 10 minutes before use and allowed to dry. This was repeated three times with *B. cenocepacia* J2315 and the average CFU/ml was reported.

3.3.2.1.2 TVC Diluent

The starting inoculum (10 ml of ~1x10^9 CFU/ml of *B. cenocepacia* J2315 culture grown in TSA broth and incubated at 37°C for 18 h) was centrifuged and washed three times in phosphate-buffered saline (PBS). The pelleted culture was then resuspended in 1ml of PBS and added to shampoo and the PBS control in a 1/10 ratio. This inoculated shampoo was serially diluted in two different diluents:0.1% peptone solution and 0.1% peptone and 2% Tween 80 solution and plated out onto the 7 media combinations. The PBS control was serially diluted in PBS. This was repeated three times with *B. cenocepacia* J2315 and the average CFU/ml was reported.

3.3.2.1 Broad Range Detection Methodology

The broad range detection protocol can be separated into four sections: sample treatment, organism capture, and nucleic acid extraction, amplification and visualisation methods (Figure 2). Two distinct methodologies were devised for organism capture: high speed centrifugation and filtration. All four sections of the detection protocol underwent optimisation experiments. All experiments were repeated in triplicate with different Bcc strains to examine utility across Bcc contaminants.

3.3.2.1.1 Sample Treatment

To simulate a contaminated product, shampoo was inoculated in a 1/10 ratio with approximately 1x10^9 CFU/ml of Bcc culture. The inoculated shampoo sample was serially diluted in a diluent and left for 30 minutes to allow bacterial recovery.

3.3.2.1.1.1 Optimisation of Sample Treatment: Addition of Preservative Neutralisers to Diluent

To investigate if the addition of neutralisers, such as Tween 80, enhanced molecular methods for bacterial detection or if dilution effects were sufficient to negate the presence of preservatives in the shampoo, inoculated shampoo samples were diluted in 0.1% Peptone and 0.1% Peptone with 2% Tween 80, the centrifugation protocol and filtration protocols were performed as outlined in sections 3.3.2.1.2.1 and 3.3.2.1.2.2 followed by RNA extraction, amplification and visualisation as outlined

in sections 3.3.2.1.3.1 and 3.3.2.1.4. This was repeated in four different shampoo matrices with the Bcc strain *B. cenocepacia* J2315.

3.3.2.1.1.2 Optimisation of Sample Treatment: Use of High Concentration Preservative Neutralisers in Diluent

In most QC microbiology protocols, samples are diluted in a 2% Tween 80 diluent however pharmacopeia enables the use of 20% Tween 80 diluent. In order to determine if increasing the concentration of Tween 80 in diluent increases detection sensitivity 2%, 5%, 10% and 20% of Tween 80 was added to 0.1% Peptone and examined as a sample diluent. The filtration protocol was performed as outlined in sections 3.3.2.1.2.2, followed by DNA extraction, amplification and visualisation as outlined in sections 3.3.2.1.3.2 and 3.3.2.1.4. This was repeated twice in one shampoo matrix with the Bcc strain *B. cenocepacia* J2315.

3.3.2.1.1.3 Optimisation of Sample Treatment: Increasing the Volume of Diluent

In traditional QC microbiology samples are diluted in a ratio of 1/10 however increasing them further should dilute out the inhibitory substances found in shampoo. Therefore 1/100, 1/1000 and 1/10000 dilution ratios were also used. Serial dilutions of bacterial cell culture were added to these diluted shampoo samples. The filtration protocols were performed as outlined in sections 3.3.2.1.2.2, followed by DNA extraction, amplification and visualisation as outlined in sections 3.3.2.1.3.2 and 3.3.2.1.4. This was repeated four times in two shampoo matrices with the Bcc strain *B. lata* 1554.

3.3.2.1.2 Organism Capture Methodologies

Organism capture was performed using two methodologies: Centrifugation and filtration.

3.3.2.1.2.1 Organism Capture by Centrifugation Method

Samples were transferred to sterile 15 ml glass Corning tubes. The tubes were parafilm sealed and then centrifuged at 5,000 x g for 10 minutes. 90% of the supernatant was removed and then bacterial pellet and supernatant was removed and

added to a 1.5 ml Eppendorf tube. Samples were made up to 1 ml with diluent and centrifuged for 10 minutes at 5,000 x g.

3.3.2.1.2.1.1 Optimisation of Centrifugation Method: Centrifugation Speed

To test the effect of centrifugation speed on detection sensitivity a 9 g shampoo sample was inoculated with 1 ml of *B. cenocepacia* strain J2315 (approximately 10^9 CFU/ml) and this was diluted 1/10 in 0.1% peptone and 2% Tween 80 and eight 10 ml aliquots were taken as samples. Four samples were centrifuged at 5,000 x g and four were centrifuged at 10,000 x g. The centrifugation protocol (section 3.3.2.1.2.1) and RNA extraction protocol (3.3.2.1.3.1) were undertaken as previously described. RNA yields were compared using a Nanodrop Spectrophotometer (ThermoFisher, Massachusetts, USA). This was repeated three times in one shampoo matrix with the Bcc strain *B. cenocepacia* J2315.

3.3.2.1.2.1.2 Optimisation of Centrifugation Method: Centrifugation Volume

To test the effect of volume of centrifuged sample on detection sensitivity a 9 g shampoo sample was inoculated with 1 ml of *B. cenocepacia* strain J2315 (approximately 10^9 CFU/ml), this was diluted 1/10 in 0.1% peptone and 2% Tween 80. Four 10 ml aliquots and four 15 ml aliquots were taken as samples, and centrifuged at 10,000 x g. The centrifugation protocol (section 3.3.2.1.2.1) and RNA extraction protocol (3.3.2.1.3.1) were undertaken as previously described. RNA yields were compared using a Nanodrop Spectrophotometer (ThermoFisher, Massachusetts, USA). This was repeated three times in one shampoo matrix with the Bcc strain *B. cenocepacia* J2315.

3.3.2.1.2.2 Organism Capture by Filtration Methodology

Samples were filtered through a 2.5 µm cellulose nitrate filter (Pall Life Sciences, New York, USA) using a Millipore glass filter-housing and funnel (Darmstadt, Germany) and a Vaccubrand diaphragm pump (Wertheim, Germany). This procedure was carried out in a Class 2 biological safety cabinet under sterile conditions. Cellulose nitrate filters were carefully transferred to sterile universals filled with 5 ml of 0.1% peptone + 2% Tween 80 solution. Universals were then shaken at 300 rpm for 30 minutes at room temperature. The diluent was transferred

to a 2 ml sterile Eppendorf tube and centrifuged at 21000 x g for 10 minutes and the supernatant removed, this was repeated until all the liquid had been removed.

3.3.2.1.2.2.1 Optimization of the Filtration Protocol: Increased Agitation of Bacterial Cells from Filter

To increase bacterial cell agitation off the filter and into the diluent, 0.5 g of sterile glass beads were added to universals. The filtration protocol (section 3.3.2.1.2.2) and RNA extraction protocol (3.3.2.1.3.1) were undertaken as previously described. RNA yields were compared using a Nanodrop Spectrophotometer (ThermoFisher, Massachusetts, USA). This was repeated three times in three different shampoo matrices with the Bcc strain *B. cenocepacia* J2315.

3.3.2.1.3 Nucleic Acid Extraction

DNA or RNA was extracted from samples as described below.

3.3.2.1.3.1 RNA Extraction Protocol

The bacterial pellet was reconstituted in 1 ml of Qiagen RNAlater stabilisation solution (Limburg, Netherlands) and incubated overnight at 4^{o}C. The Qiagen RNeasy mini kit (Limburg, Netherlands) extraction protocol was followed. cDNA libraries were created from extracted RNA using the Bioline Tetro cDNA kit (London, UK), following manufacturer's instructions. A positive control (*B. cenocepacia* J2315 RNA extracted and quantified from pure culture) and a negative control were included in the cDNA protocol.

3.3.2.1.3.2 DNA Extraction Protocol

The Qiagen DNeasy mini kit (Limburg, Netherlands) extraction protocol was followed as per the manufacturer's instructions. As per the protocol, samples were incubated for 1 hr at 56^{o}C with protinase K.

3.3.2.1.3.3 Optimization of the Nucleic Acid Extraction Protocol: Comparison of DNA and RNA

To compare DNA and RNA yields obtained from both protocols, the centrifugation and filtration protocols were performed in duplicate as outlined in sections

3.3.2.1.2.1 and 3.3.2.1.2.2, and one set of samples followed the RNA extraction protocol as outlined in section 3.3.2.1.3.1, and one set of samples followed the DNA extraction protocol as outlined in section 3.3.2.1.3.2. Nucleic acid was quantified using Invitrogen Qubit Fluorometric analysis (California, USA) and PCR amplified and visualised on agarose gel as outlined in sections 3.3.2.1.4. This was repeated twice in one shampoo matrix with the Bcc strain *B. cenocepacia* strain 1558.

3.3.2.1.4 Amplification and Visualisation of Nucleic Acid

Nucleic acid was PCR amplified using universal 16S rRNA gene primers 27f and 1492r which spans the V1-V9 regions (Lane, 1991) and Qiagen HotStarTaq Plus Master Mix Kit (Limburg, Netherlands). PCR products were visualised using a UV transilluminator, on a 1% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany), using electrophoresis techniques.

3.3.2.1.4.1 Optimization of Amplification and Visualisation of Nucleic Acid: Input Volume in PCR

In order to increase the likelihood of low-level DNA amplification, triplicate PCR's were run with 2 µl, 5 µl and 10 µl of extracted DNA. Samples were processed using the filtration protocol (outlined in section 3.3.2.1.2.2) and DNA extraction and end point PCR methods were as outlined in sections 3.3.2.1.3.2 and 3.3.2.1.4. This was repeated twice in one shampoo matrix with the Bcc strain *B. cenocepacia* J2315.

3.3.2.1.4.2 Optimization of Amplification and Visualisation of Nucleic Acid: Use of SYBR Gold Gel Stain

To increase the detection of nucleic acids, Invitrogen's SYBR gold nucleic acid gel stain (California, USA) was used. The SYBR gold was diluted in TAE to give a 1X strength solution. The pH of the solution was checked to be within the optimal staining range. The gel was submerged into the buffer solution, covered using foil and gently agitated at room temperature for 40 minutes. The gel was visualized using 300 nm UV transilluminator and images were taken using a CCD camera. This was performed with the filtration protocol (section 3.3.2.1.2.2) with RNA and DNA extracted and amplified as outlined in sections 3.3.2.1.3.1, 3.3.2.1.3.2 and 3.3.2.1.4. This was repeated twice in one shampoo matrix with the Bcc strain *B. lata* 1554.

3.3.2.1.4.2 Optimization of Amplification and Visualisation of Nucleic Acid: qPCR

To increase the sensitivity of detection, quantitative PCR (qPCR) amplification was performed on the DNA extracted from samples using the filtration protocol as outlined in sections 3.3.2.1.2.2 and 3.3.2.1.3.2. The creation and preparation of the qPCR plasmid standard, and qPCR parameters are as follows. To create the qPCR plasmid standard the 16s region of *B. cenocepacia* J2315 was PCR amplified using Qiagen HotStarTaq Plus Master Mix Kit (Limburg, Netherlands). This was then ligated into the pCR 2.1 plasmid and transformed into competent *E. coli* Top10 cells using the TA Cloning Kit by Invitrogen (California, USA) as per the manufacturer's instructions. Positive clones were identified by X-Gal/IPTG screening and PCR amplification using M13 region targeting primers (primers are given in Table 6). Long term stocks of positive purified clones were made by inoculating single colonies into 2 ml of LB containing 50 µg/ml Kanamycin and growing overnight and adding 15% sterile glycerol and transfer to a cryovial at -80°C. Plasmid was isolated from bacterial cells using the isolation of high copy plasmid DNA protocol from the Bioline plasmid mini kit (London, UK). 4 µl of plasmid extraction was visualized on a 1% agarose gel. To linearize supercoiled plasmid, plasmid ligation sites were identified, and restriction site mapping software Webcutter 2.0 (**http://rna.lundberg.gu.se/cutter2/**) was used to ensure ligation sites were not identified in qPCR primer binding regions of all six copies of the *B. cenocepacia* J2315 16srRNA sequence. Three suitable non-interfering double cutter positions were identified, Nsi1, Xba1, Xho1. All three enzymes (New England Biolabs) were used in ligation reactions containing 12.5 µl water, 0.5 µl of enzyme, 5 µl of supercoiled plasmid and 2 µl NEBuffer 2.1 and incubated at 37 °C for one hour and visualized on a 1% agarose gel. Xho1 gave the best ligation results (complete digestion and clear bands) and was therefore used in subsequent ligation reactions. Ligation reactions were incubated with Xho1 at 85ºC for 10 minutes and cleaned up using the Bioline PCR clean up kit (London, UK). Plasmid DNA was then Qubit flurometric analysis quantified and serially diluted in Qiagen nuclease free water (Limburg, Netherlands) to give a standard curve. qPCR reactions were set up in 96 well plates (Grenier Bio-one, UK) and run in the StepOnePlus Real Time qPCR System (ThermoFisher Scientific, Massachusetts, United States). Each 25 µl qPCR reaction contained 12.5 µl of Bioline Sensimix SYBR with High ROX (London,

UK), 6 µl of nuclease free water, 1 µl of 250 nM forward primer, 1 µl of 250 nM reverse primer and 5µ of sample. Standards, samples and no template controls were run in triplicate on the same plate. The thermocycler conditions were as follows: 1 cycle of 95 °C for 10minutes, 40 cycles of 95 °C for 15s, 60 °C for 15s and 72 °C for 15s. This was repeated five times.

3.3.2.2 Narrow Range Detection Methodology

Due to the requirement of an easy and quick detection protocol, a simple PCR based detection protocol was designed which involved only two steps: sample treatment and nucleic acid amplification and visualisation (Figure 2). Both sections of this protocol underwent optimisation experiments. The boil prep methodology as outlined in sections 3.3.2.2.1 and 3.3.2.2.2 were repeated ten times with two different Bcc strains: *B. cenocepacia* J2315 and *B. cenocepacia* 1558.

3.3.2.2.1 Sample Treatment

Shampoo was diluted to 1/1000 in sterile water. Bcc strains were cultured for 18 hours in 3 ml of BSM at 30°C and serially diluted in the shampoo and water to give a concentration gradient from ~1 x 10^8 CFU/ml to ~1 x 10^3 CFU/ml. 1 ml of each dilution was boiled at 95°C for 10 minutes and then centrifuged at 1500 x g for 10 minutes.

3.3.2.2.1.1 Optimisation of Sample Treatment for Boil Prep Methodology: Diluent

To investigate if the addition of neutralisers, such as Tween 80, enhanced molecular methods for bacterial detection and if increasing the concentration of Tween 80 in diluent increases detection sensitivity a 1/1000 dilution of shampoo was made in three different diluents: 0.1% peptone + 2% Tween (N=10), 0.1% peptone + 5% Tween (N=6) and 0.1% peptone + 10% Tween (N=3). The boil prep methodology was performed as outlined in 3.3.2.2.1 and PCR amplification and amplicon visualisation were performed as outlined in 3.3.2.2.2. This was replicated nine times with two different Bcc strains: *B. cenocepacia* J2315 and *B. cenocepacia* 1558.

3.3.2.2.1.2 Optimisation of Sample Treatment for Boil Prep Methodology: Lysis Buffer

To examine the effect of an additional lysis buffer treatment of samples prior to the boil prep methodology 100 µl of each dilution was added to 200 µl of lysis buffer solution containing; Tris 1 mM, 0.1mM EDTA, 3 µl of 20 mg/ml Proteinase K, and 0.5% Tween 20 as described in (Samadi *et al.,* 2007). The samples were incubated for 60 minutes at 35ºC, followed by a 10-minute incubation at 95 °C. The boil prep methodology was performed as outlined in 3.3.2.2.1 and PCR amplification and amplicon visualisation were performed as outlined in 3.3.2.2.2. This was replicated nine times with two different Bcc strains: *B. cenocepacia* J2315 and *B. cenocepacia* 1558.

3.3.2.2.2 Nucleic Acid Amplification and Visualisation

10 µl was used as sample template for end point PCR's with *Burkholderia* Specific primers (See Table 6). Each 50 µl PCR reaction contained 25 µl of Bioline BioMix Red Master Mix (London, UK), 11 µl of nuclease free water, 2 µl of 250 nM forward primer, 2 µl of 250 nM reverse primer and 10 µl of sample. The thermocycler conditions were as follows: 1 cycle of 95 °C for 5 minutes, 35 cycles of 94 °C for 1 minute, 60 °C for 45s and 72 °C for 45s and 72 °C for 10 minutes. PCR products were visualised using a UV transilluminator, on a 1% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany) using electrophoresis techniques.

3.3.2.2.2.1 Optimisation Amplification and Visualisation of Nucleic Acid for Boil Prep Methodology: Input Volume in PCR

In order to increase the likelihood of low-level DNA amplification, 0.5 µl, 2 µl, and 10 µl of sample was added to PCR reactions, and bands were visualised and compared as outlined 3.3.2.2.2. This was replicated nineteen times with two different Bcc strains: *B. cenocepacia* J2315 and *B. cenocepacia* 1558.

3.3.2.2.2.1 Optimisation Amplification and Visualisation of Nucleic Acid for Boil Prep Methodology: Lysis buffer treatment and Nested PCR

In order to increase low level detection a lysis buffer treatment and nested PCR approach was examined. Bcc strains were cultured for 18h in 3 ml of BSM at 30°C

(approximately ~1 x 10^8 CFU/ml) and added 1/10 to neat shampoo to simulate contaminated samples. The lysis buffer treatment was followed as outlined in 3.3.2.2.1.2 . PCR reactions were set up as previously described with broad range universal primers 27f and 1492r with the extension temperature of 40 °C (Table 6). First round PCR products were visualised on 1% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany). First round PCR products were serially diluted in sterile water 1/1000, and 10 µl was added to second round PCR reactions with *Burkholderia* specific primers with an extension temperature of 60°C (Table 6). Second round PCR products were visualised as previously described in section 3.3.2.2.2. This was repeated three times with *B. cenocepacia* 1558.

3.3.3 Assessing Method Utility on Archived Contamination Samples

Unilever provided 17 archived personal care samples which had been identified as contaminated between 2002-2014. Traditional TVC's were performed to estimate total bioburden (outlined in section 2.4.3) and the lysis buffer and nested boil prep-based PCR detection methodology (outlined in section 3.3.4.1). PCR's were amplified using Qiagen Hot Star Taq (Limburg, Netherlands) according to the manufacturer's instructions and the total volume for each reaction was 50 µl. First round end point PCR was performed with 27f and 1492r universal bacterial primers and visualised with 1% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany) and Bioline Hyperladder 1KB molecular ladder (London, UK). Second round end point PCR was performed at 60°C with universal primers, *Burkholderia* specific primers and *Pseudomonas* specific primers (see table 6). PCR products were visualised with 3% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany) and Bioline Hyperladder IV ladder (London, UK).

3.4 Results

3.4.1 Method Development Using Bacterial Cultures

3.4.1.1 Optimisation of RNA Protocols

To reduce protocol time within the ideal "7-hour shift" the shortening of the current Qiagen RNA extraction kit protocol (Limburg, Netherlands) was investigated by reducing the incubation period with the RNA stabilisation agent "RNAlater". Four Bcc strains (*B. cepacia* ATTC25416, *B. vietnamesis* pc279, *B. multivirons* c5393 and *B. dolosa* bcc232) were grown in 5 ml LB broth at 37°C for 12 h to give a mid-log culture approximately 1 x 10^6 CFU/ml. Duration of RNAlater incubation was detrimental to RNA yield. Reducing the incubation time from 24 h to 4h resulted in a minimum of a 50% reduction in RNA yield (Figure 3).

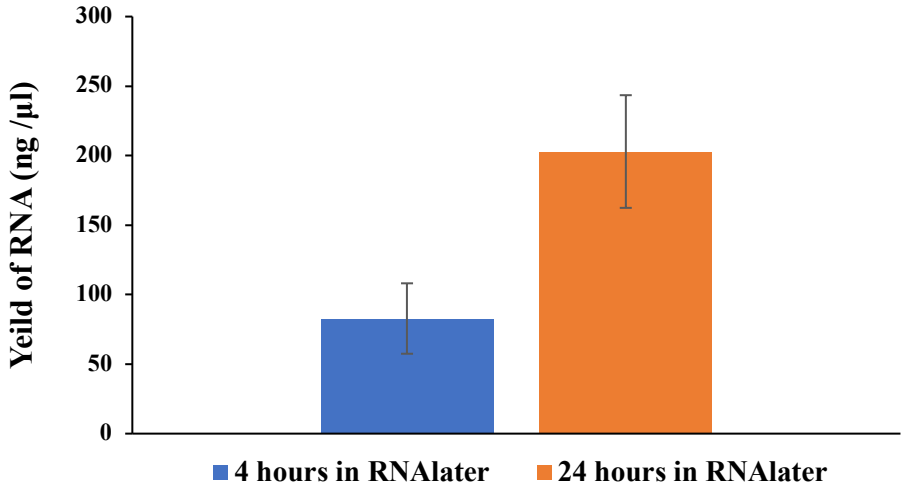


Figure 3: The effect of Qiagen RNAlater incubation time on the mean yield of RNA from four Bcc strains using Qiagen RNeasy extraction protocol. Qiagen RNAlater is an RNA stabilisation solution which was used with either a 24hr or 4hr incubation period. Error bars show the standard error for four biological replicates.

3.4.1.2 Comparison of DNA and RNA yields

The relative RNA and DNA yields recovered from cultures of four Bcc strains grown in LB nutrient medium at 37 °C for 12 hours at a concentration of approximately 1 x 10^6 CFU/ml were investigated. Results showed a higher yield of RNA isolated from parallel samples than DNA. This phenomenon was seen across all four Bcc genomovars indicating reproducibility of results (Figure 4). RNA is

more abundant in the cell and should be a much better target for detection than DNA. However, as the relative efficiencies of the RNA and DNA extraction kits are unknown it is possible (but unlikely) that this result could also be caused by the efficiency of the DNA and RNA extraction method used.

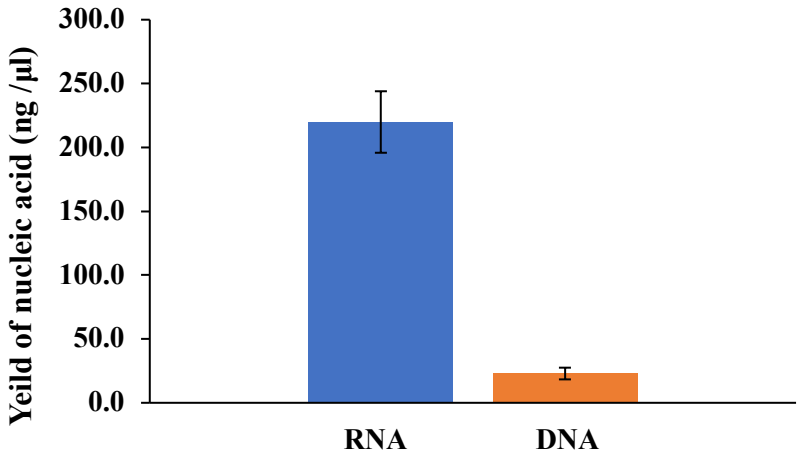


Figure 4: The mean yield of nucleic acid (RNA and DNA) from *B. cepacia* ATTC 25416 and *B. cenocepacia* J2315. Error bars show the standard error for six technical replicates from two biological replicates.

3.4.1.3.1 Viability PCR Methods - Ethidium monoazide PCR (EMA-PCR)

Ethidium monoazide is a DNA stain which selectively inhibits PCR amplification of DNA from non-viable cells. Viable and non-viable cells were treated with 10 µl/ml ethidium monoazide followed by DNA extraction, end point PCR using 16s rRNA primers, PCR product purification and quantification by Qubit flurometric analysis as described in 3.3.1.3.1. Mean concentrations of DNA amplified from viable and non-viable cells during EMA-PCR did not significantly differ (Figure 5). Statistical analysis of the small sample size (N=3) using an unpaired t-test result confirmed no statistical significance between viable and non-viable cells (*P*= 0.3209).

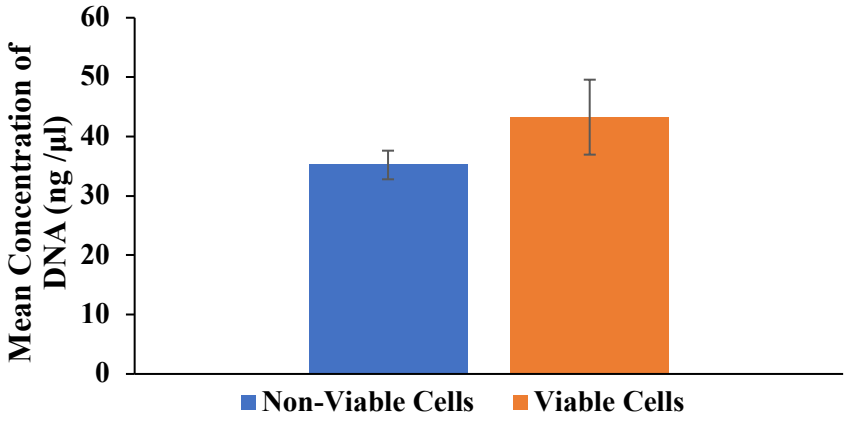


Figure 5: Mean concentration of PCR amplified DNA from viable and non-viable cells of *B. cenocepacia* strain 1558 treated with 10 µl/ml ethidium monoazide (EMA). Error bars show the standard error for three technical replicates.

Crude analysis using electrophoresis visualisation of serially diluted DNA of non-viable cells with EMA treatment and without EMA treatment resulted in the same detection sensitivity (10^{-3}) (Figure 6). Treatment with EMA also negatively impacted visualisation of DNA from viable cells, with reduced band brightness and a 10-fold reduction in detection sensitivity for DNA extracted from viable cells treated with EMA (10^{-3}) than DNA from viable cells not treated with EMA (10^{-4}). DNA extracted from both viable and non-viable cells without EMA treatment showed stronger bands than the viable/non-viable cells treated with EMA. As seen in the gel images displayed in figures 5 A and B, DNA extracted from both viable and non-viable cells with EMA treatment was not detectable lower than 10^{-3} as visualised by no detectable bands on the gel, indicating the DNA concentration 10^{-3} is the threshold for accurate detection.

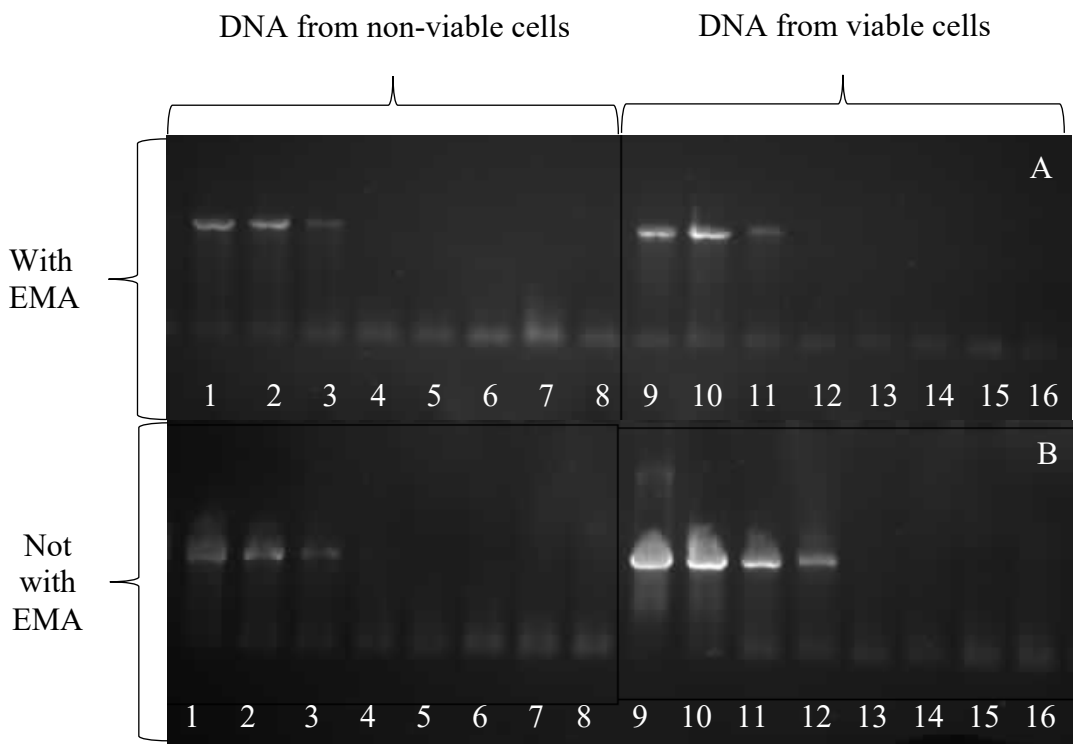


Figure 6*:* Viable and non-viable *B. cenocepacia* strain 1558 cells treated with and without 10 µl/ml EMA prior to DNA extraction. DNA was serially diluted from 10^{-1} to 10^{-8} and DNA dilutions were then used in end point PCR using universal 16s rRNA primers (27f and 1492r). Amplification products (1465bp) were visualized on a 1% agarose gel with Midori green DNA stain using electrophoresis. A) EMA treatment: Non-viable cells (Lanes 1-8), viable cells (Lanes 9-15). B) Without EMA treatment: Non-viable cells (Lanes 1-8), viable cells (Lanes 9-15).

3.4.1.3.2 Optimisation of EMA protocols

In order to investigate optimal EMA treatment concentration, viable and non-viable bacterial cultures were treated with 5 µl/ml and 7.5 µl/ml of EMA. EMA-PCR was performed as previously described in 3.3.1.3.1 with the exception that extracted DNA was not diluted for PCR amplification. Crude analysis of gel electrophoresis imaging of EMA PCR amplicons found no significant difference in DNA abundance (as estimated by band width and brightness) of non-viable or viable cells treated with 5 µl/ml and 7.5 µl/ml of EMA (Figure 6). Visual estimates of DNA extracted from EMA treated non-viable cells with 5 µl/ml and 7.5 µl/ml of EMA solution was comparable to DNA extracted from the control (non-viable cells without EMA treatment). This demonstrates that EMA treatment did not inhibit PCR amplification of dead cells as expected. Furthermore, DNA extracted from EMA treated viable

cells with 5 µl/ml and 7.5 µl/ml EMA solution is lower (as estimated by band width and brightness) than extracted DNA from viable cells without EMA treatment, demonstrating an under-representation of viable cell count.

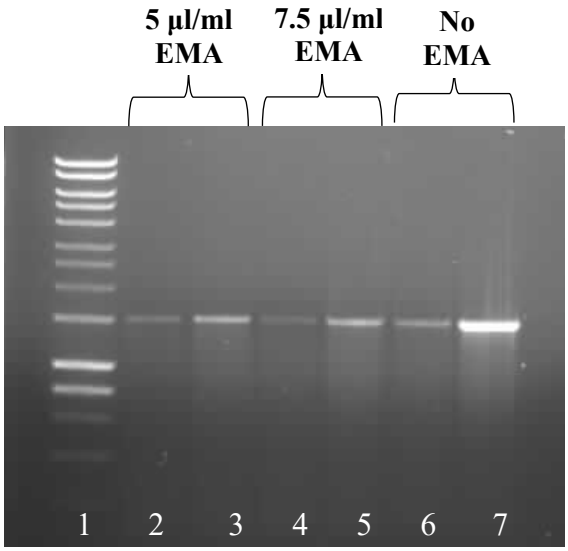


Figure 7*:* DNA extracted from *B. cenocepacia* strain 1558 viable and non-viable cells treated with 5 µl/ml and 7.5 µl/ml prior to DNA extraction. Extracted DNA was used in end point 16s rRNA PCR with universal primers (27f and 1492r). Amplification products (1465bp) were visualized on a 1% agarose gel with Midori green DNA stain using electrophoresis. Gel image shows: Lane 1: Bioline Hyperladder 1 with a size range of 200 - 10,037bp, Lane 2: Non-viable cells with 5 µl/ml EMA, Lane 3: Viable cells with 5 µl/ml EMA, Lane 4: Non-viable cells with 7.5 µl/ml EMA, Lane 5: viable cells with 7.5 µl/ml EMA, Lane 6: Control, non-viable cells without EMA, Lane 7: Control, viable cells without EMA.

3.4.2 Method Development using Artificially Contaminated Personal Care Products

3.4.2.1 Total Viable Counts

The optimal diluent/media for the recovery of stressed bacterial contaminants was investigated. *B. cenocepacia* J2315 culture grown in TSA broth and incubated at 37°C for 18 h at a concentration of approximately 1 x 10^6 CFU/ml was washed and resuspended in PBS to remove growth media. This culture was used to inoculate a PBS control (without shampoo) and shampoo sample in a 1/10 ratio. The shampoo sample was then serially diluted in 0.1% peptone solution and 0.1% peptone and 2% Tween 80 solution and left for 30 minutes to allow bacterial recovery. The PBS

control was serially diluted in PBS and left for 30 minutes to allow bacterial recovery. These were plated out onto 7 media combinations: TSA, R2A, R2A + 0.01% Bovine Catalase, 50% R2A + 50% LB Agar, 50% R2A + 50% LB Agar + 0.01% Bovine Catalase, LB Agar, LB + 0.01% Bovine Catalase (Sigma Aldrich, Missouri USA). This was repeated three times with *B. cenocepacia* J2315 and the average CFU/ml was reported.

3.4.2.1.1 TVC Medium Design

Enumeration of artificially contaminated shampoo on all media combinations resulted in ≥70% recovery of the starting inoculum's calculated value, which is the defined limit of an acceptable growth promotion media according the FDA Pharmaceutical Microbiology Manual (FDA, 2014). However, there was no enhancement of growth (calculated as TVC's) for one of the 6 nutrient depleted media over the standard nutrient rich media TSA (Table 7).

3.4.2.1.2 TVC Diluent

There was a log difference for the use of 0.1% peptone and 2% Tween 80 rather than 0.1% peptone alone (Table 7). This is also seen with molecular data (Figure 8) whereby the use of Tween neutralisers increased PCR sensitivity of Bcc amplification from shampoo. Mean total viable counts (calculated as CFU/ml) of *B. cenocepacia* J2315 diluted into shampoo were lower when compared to the PBS control. *B. cenocepacia* J2315 diluted in PBS (as a no shampoo control) produced TVCs of 10^8 CFU/ml, while TVCs from 1% shampoo diluted in 0.1% peptone produced TVC's 4 log lower than those from PBS, and TVCs from 1% shampoo with 0.1% peptone and 2% Tween 80 produced TVC's 3 log lower than those from PBS. This demonstrates the toxic effect of shampoo on viable bacterial load.

The reported TVC's are for 1% shampoo concentrations (1/100 dilution factor). 10% shampoo concentrations were investigated but resulted in poor bacterial recovery and inaccurate estimates (Data not shown). This demonstrates that antimicrobial substances in the shampoo interfere with the cultivation of bacteria and further confirms the need of neutralisers such as Tween 80.

Table 7: Bacterial recovery of *B. cenocepacia* J2315 from serial dilutions using PBS, 1% Shampoo with 0.1% Peptone and 1% Shampoo with 0.1% Peptone, 2% Tween 80 plated and grown overnight on 7 different agars with supplements. This was repeated in triplicate and the averages reported.

	PBS	1% Shampoo with 0.1% Peptone	1% Shampoo with 0.1% Peptone, 2% Tween 80
TSA	7.5 x 10^8 CFU/ml	4.7 x 10^4 CFU/ml	2.4 x 10^5 CFU/ml
LB	5.7 x 10^8 CFU/ml	2.1 x 10^4 CFU/ml	1.9 x 10^5 CFU/ml
LB + Catalase	7.0 x 10^8 CFU/ml	7.8 x 10^4 CFU/ml	4.4 x 10^5 CFU/ml
R2A	7.2 x 10^8 CFU/ml	5.2 x 10^4 CFU/ml	3.8 x 10^5 CFU/ml
R2A + Catalase	5.7 x 10^8 CFU/ml	3.4 x 10^4 CFU/ml	2.9 x 10^5 CFU/ml
LB + R2A	5.8 x 10^8 CFU/ml	4.8 x 10^4 CFU/ml	4.1 x 10^5 CFU/ml
LB + R2A + Catalase	5.4 x 10^8 CFU/ml	6.0 x 10^4 CFU/ml	5.4 x 10^5 CFU/ml

3.4.2.1 Broad Range Detection Methodology

The broad range detection protocol encompassed four distinct steps; sample treatment, organism capture (by centrifugation or filtration), nucleic acid extraction and amplification and visualisation methods. All four sections of the detection protocol underwent optimisation experiments. All experiments were repeated in triplicate with different Bcc strains to examine utility across Bcc contaminants.

3.4.2.1.1 Sample Treatment

To simulate a contaminated product, shampoo was inoculated in a 1/10 ratio with approximately 1x10^9 CFU/ml of Bcc culture. The inoculated shampoo sample was serially diluted in a diluent and left for 30 minutes to allow bacterial recovery.

3.4.2.1.1.1 Optimisation of Sample Treatment: Addition of Preservative Neutralisers to Diluent

The inoculated shampoo sample was serially diluted in 0.1% peptone diluent and 0.1% peptone with 2% Tween polysorbate 80 diluent. The centrifugation protocol and filtration protocols were performed as outlined in sections 3.3.2.1.2.1 and 3.3.2.1.2.2 followed by RNA extraction, amplification and visualisation as outlined in sections 3.3.2.1.3.1 and 3.3.2.1.4. Molecular detection of *B. cenocepacia* J2315 using both centrifugation and filtration protocols confirmed that the addition of 2% Tween polysorbate 80 to diluents increased molecular detection up to 100-fold,

when compared to 0.1% peptone diluent alone (Figure 8). This was seen previously in preliminary diluent experiments, where the addition of 2% Tween 80 to diluents used in TVC's increased estimations of bacterial load counts from artificially contaminated shampoo (Table 7).

It was found for both methodologies that the addition of 2% Tween 80 increased the sensitivity of detection between 10 and 1000-fold (Fig 8 visualises a 100-fold increase). As the quantification method was visualisation by gel electrophoresis, the improved sensitivity when using 0.1% peptone + 2% Tween 80 solution could only be crudely estimated by the visualisation of bands at lower serial dilutions. Also, the 0.1% peptone + 2% Tween 80 bands were thicker than the 0.1 % peptone solution, which also suggests an increased RNA yield (Figure 8).

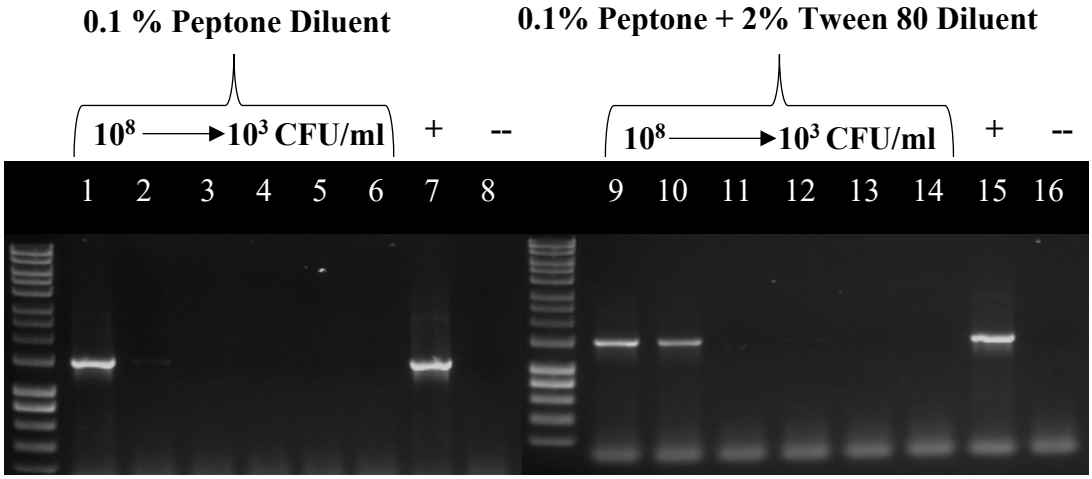


Figure 8: Detection of *B. cenocepacia* strain J2315 spiked into shampoo and serially diluted in 0.1% peptone solution (Lanes 1-6) and 0.1% peptone and 2% Tween 80 solution (Lanes 9-14). Diluted shampoo samples were centrifuged and RNA was extracted. Extracted RNA was reverse transcribed into cDNA and end-point PCR amplified using universal primers (27f and 1492r). Amplification products (1465bp) were visualised on a 1% agarose gel with Midori green DNA stain. The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp. The CFU/ml decreases tenfold from 4.51 x 10^8 CFU/ml (lanes 1 and 5) to 4.51 x 10^3 CFU/ml (lanes 4 and 8). Lane 8 and 16 : Negative PCR controls were PCR mixtures with primers, but with no added cDNA. Lanes 7 and 15: Positive experiment and PCR controls were *B. cenocepacia* strain J2315 culture without shampoo processed as above.

3.4.2.1.1.2 Optimisation of Sample Treatment: Use of High Concentration Preservative Neutralisers in Diluent

The inoculated shampoo sample was serially diluted in 0.1% peptone + 2% Tween 80 and 0.1% peptone + 5% Tween 80. The filtration protocol was performed as outlined in sections 3.3.2.1.2.2, followed by DNA extraction, amplification and visualisation as outlined in sections 3.3.2.1.3.2 and 3.3.2.1.4. Increasing the Tween polysorbate 80 concentration from 2% to 5% decreased the yield of DNA extracted from shampoo spiked with *B. cenocepacia* strain 1558 (9.31 ng/ml and 1.13 ng/ml respectively) and also decreased the sensitivity of detection by PCR amplification of 16srRNA gene PCR tenfold (Figure 9). The addition of Tween 80 above 5% resulted in solubility issues, to negate this 10% and 20% Tween 80 was heated and agitated to dissolve however this precipitated back out at normal room temperatures. Furthermore, during the filtration protocol, shampoo diluted in 10% and 20% Tween 80 blocked the 2.5 µm cellulose nitrate filter and could not be used.

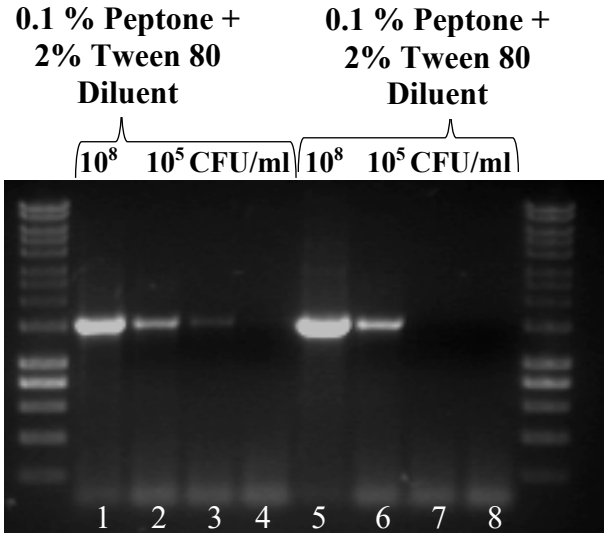


Figure 9*:* Detection of *B. cenocepacia* strain J2315 spiked into shampoo and serially diluted in diluted in 0.1% peptone and 2% Tween 80 (Lanes 1-4) and 0.1% peptone and 5 % Tween 80 (Lanes 5-8). Diluted shampoo samples were filtered and DNA was extracted. Extracted DNA was amplified using universal primers 27f/1493r and amplification products were visualized on a 1% agarose gel with Midori green DNA stain using electrophoresis. The CFU/ml decreases tenfold from 6.30 x 10^8 CFU/ml (lanes 1 and 5) to 6.30 x 10^5 CFU/ml (lanes 4 and 8). The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp.

3.4.2.1.1.3 Optimisation of Sample Treatment: Increasing the Volume of Diluent

Serial dilutions of shampoo were performed with 1/100, 1/1000 and 1/10000 dilution ratios. Serial dilutions of bacterial cell culture were added to these diluted shampoo samples. The filtration protocols were performed as outlined in sections 3.3.2.1.2.2, followed by DNA extraction, amplification and visualisation as outlined in sections 3.3.2.1.3.2 and 3.3.2.1.4. Decreasing the shampoo concentration through serial dilution increased the sensitivity of detection. This was a linear relationship with detection sensitivity increasing with shampoo dilution and bacterial cell count (Figure 10). The second lowest shampoo concentration and the lowest bacterial cell content gave a detection sensitivity of 8.36 x 10^2 CFU/ml. Dilution effects of the inhibitory substances can be seen with a greater intensity of bands at 1/1000 and 1/10000 when compared to 1/100 or 1/10.

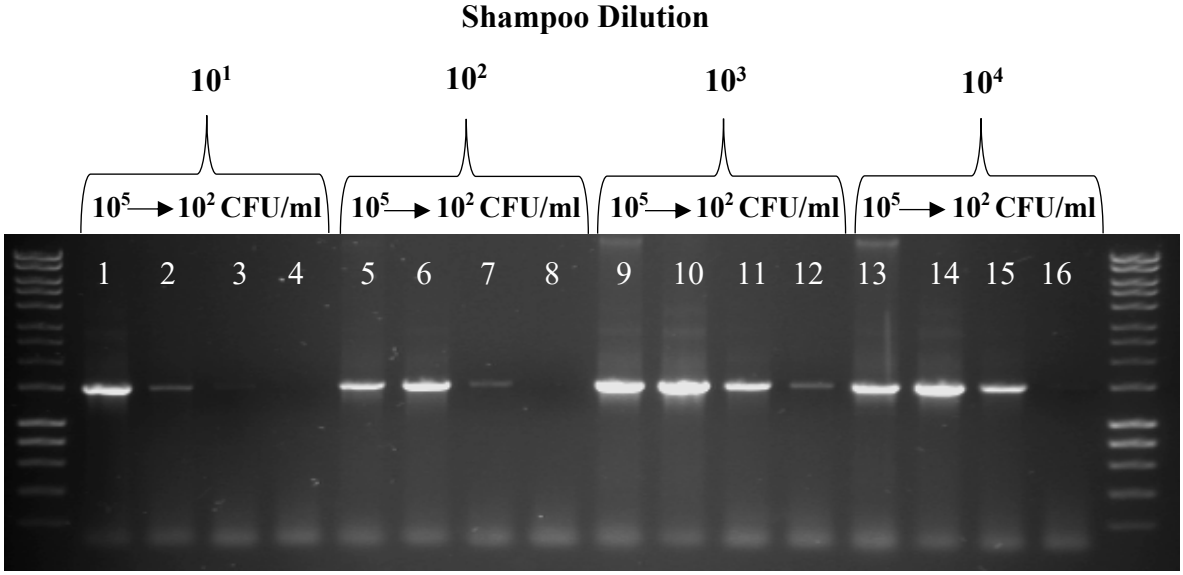


Figure 10: The effect of shampoo dilution on detection of DNA of *B. lata* strain 1554. Shampoo was diluted 10^1 (lanes 1-4), 10^2 (lanes 5-8), 10^3 (lanes 9-12) and 10^4 (lanes 13-16) and spiked with decreasing concentrations of *B. lata* strain 1554, from 8.36 x 10^5 CFU/ml (lanes 1, 5, 9 and 13) to 8.36 x 10^2 CFU/ml (lanes 4, 8, 12 and 16). Diluted shampoo samples were filtered, and DNA was extracted. Extracted DNA was amplified using universal primers 27f/1493r and amplification products were visualized on a 1% agarose gel with Midori green DNA stain using electrophoresis. The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp. The negative PCR control of PCR mixtures with primers but with no added DNA and the positive experiment/PCR control consisted of *B. lata* strain 1554 culture grown overnight and processed as experimental samples (data not shown).

3.4.2.1.2 Organism Capture Methodologies

Organism capture was performed using two methodologies: Centrifugation and filtration.

3.4.2.1.2.1 Organism Capture by Centrifugation Method

Diluted shampoo samples were transferred to sterile 15 ml glass tubes, parafilm sealed and centrifuged at 5,000 x g for 10 minutes. 90% of the supernatant was removed and then bacterial pellet and supernatant were added to a 1.5 ml Eppendorf tube. Samples were made up to 1 ml with diluent and centrifuged for 10 minutes at 5,000 x g. The centrifugation method only achieved detection of significant bacterial contamination of 2.20 x 10^6 CFU/ml and above (Figure 11). Nanodrop results for RNA extracted by the centrifugation method showed low 260/230 and 260/280 values, indicative of the co-extraction of inhibitory substances, presumably from the bacterial pellet which could damage and reduce RNA yield.

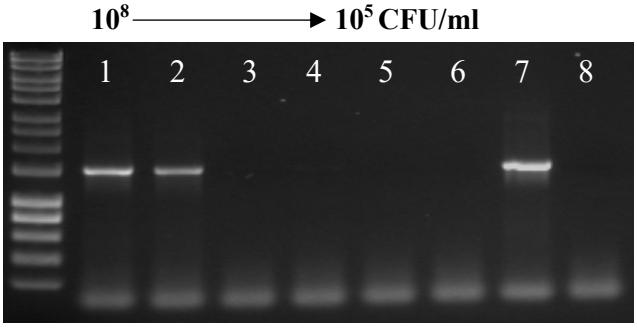


Figure 11: Detection of *B. cenocepacia* strain J2315 spiked into shampoo using a centrifuge based methodology to separate shampoo and organism cells. PCR amplified 16s region (1465bp) from RNA extraction from serial dilutions of shampoo spiked with *B. cenocepacia* using the centrifugation method. Extracted RNA was reverse transcribed into cDNA and end-point PCR amplified using universal primers (27f and 1492r). Amplification products were visualized on a 1% agarose gel with Midori green DNA stain using electrophoresis. Lanes 1-6: Serial dilutions of bacterial cells from 2.20 x 10^7 CFU/ml to 2.20 x 10^2 CFU/ml. The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp. Lane 7: The positive experiment/PCR control consisted of *B. cenocepacia* culture grown overnight and processed as experimental samples. Lane 8: The negative PCR control of PCR mixtures with primers, but with no added cDNA.

3.4.2.1.2.1.1 Optimisation of Centrifugation Method: Centrifugation Speed

Attempts to increase centrifugation speed to circumvent detergent effects and increase RNA yield did not result in increased detection sensitivity. Average RNA extraction yields measured using Qubit flurometric quantification demonstrated almost identical yield from 5,000 rpm and 10,000 rpm, 0.094 ug/ml and 0.095 ug/ml respectively.

3.4.2.1.2.1.2 Optimisation of Centrifugation Method: Centrifugation Volume

Increasing volume of the sample for centrifugation from 10 ml to 15 ml to increase low level bacterial detection did not increase detection sensitivity. Average RNA extraction yields measured using Qubit flurometric quantification showed there was no significant difference for RNA yield from 10ml aliquots (0.097 ug/ml) and 15 ml aliquots (0.082 ug/ml).

3.4.3.2.1.2.2 Organism Capture by Filtration Methodology

Samples were filtered through a 2.5 µm cellulose nitrate filter using a Millipore glass filter-housing and funnel and diaphragm pump. Cellulose nitrate filters were transferred to sterile universals filled with 5 ml of 0.1% peptone + 2% Tween 80 solution. Universals were then shaken at 300 rpm for 30 minutes at room temperature. The diluent was transferred to a 2 ml sterile Eppendorf tube and centrifuged at 21000 x g for 10 minutes and the supernatant was removed. This was repeated until all the liquid had been removed. The filtration method could only detect contamination in shampoo at bacterial loads of 3.57×10^6 CFU/ml and above (see figure 12).

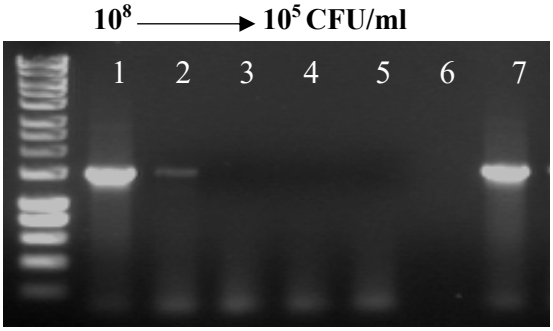


Figure 12: Detection of *B. cenocepacia* strain J2315 spiked into shampoo using a filtration based methodology to separate shampoo and organism cells. Serial dilutions of shampoo spiked with *B. cenocepacia* were processed using the filtration method and RNA extraction protocols. Extracted RNA was converted into cDNA and end-point PCR amplified using universal primers (27f and 1492r). Amplification products were visualized on a 1% agarose gel with Midori green DNA stain using electrophoresis. Lanes 1-5: Serial dilutions of bacterial cells from 3.57 x 10^7 CFU/ml to 3.57 x 10^3 CFU/ml. The molecular ladder is Bioline Hyperladder 1 with a size separation of 200 - 10,037bp. Lane 6: The negative PCR control of PCR mixtures with primers, but with no added cDNA. Lane 7: The positive experiment/PCR control consisted of *B. cenocepacia* culture grown overnight and processed as experimental samples.

3.4.2.1.2.2.1 Optimization of the Filtration Protocol: Increased Agitation of Bacterial Cells from Filter

To increase bacterial cell agitation off the filter and into the diluent, 0.5 g of sterile glass beads were added to universals. This did not increase detection above 3.57 x 10^6 CFU/ml (data not shown).

3.4.2.1.3 Nucleic Acid Extraction

To compare DNA and RNA yields organism capture protocols were performed in duplicate. One set of samples followed the RNA extraction protocol using Qiagen RNeasy mini kit and one set of samples followed the DNA extraction protocol using Qiagen DNeasy mini kit extraction protocol. Both kits were used per the manufacturer's instructions. Extracted RNA were converted into cDNA using the Bioline Tetro cDNA kit following manufacturer's instructions. Nucleic acid was quantified using Qubit Fluorometric analysis and PCR amplified and visualised on agarose gel as outlined in sections 3.3.2.1.4. This was repeated twice for both centrifugation and filtration protocols in one shampoo matrix with the Bcc strain *B. cenocepacia* strain 1558.

Simultaneous, RNA and DNA extractions from artificially spiked shampoo samples produced comparable DNA and RNA yields as measured by the Qubit Flurometer. DNA and RNA could only be detected at 2.25 x 10^8 CFU/ml, with yields of 11.4 ng/ml and 7.45 ng/ml respectively. PCR amplification of DNA and cDNA using universal bacterial primers (27f and 1492r) targeting the 16s region (1465bp) also gave comparable detection sensitivities (2.25 x 10^6 CFU/ml) as analysed by the visualisation of bands by agarose gel electrophoresis (Figure 13).

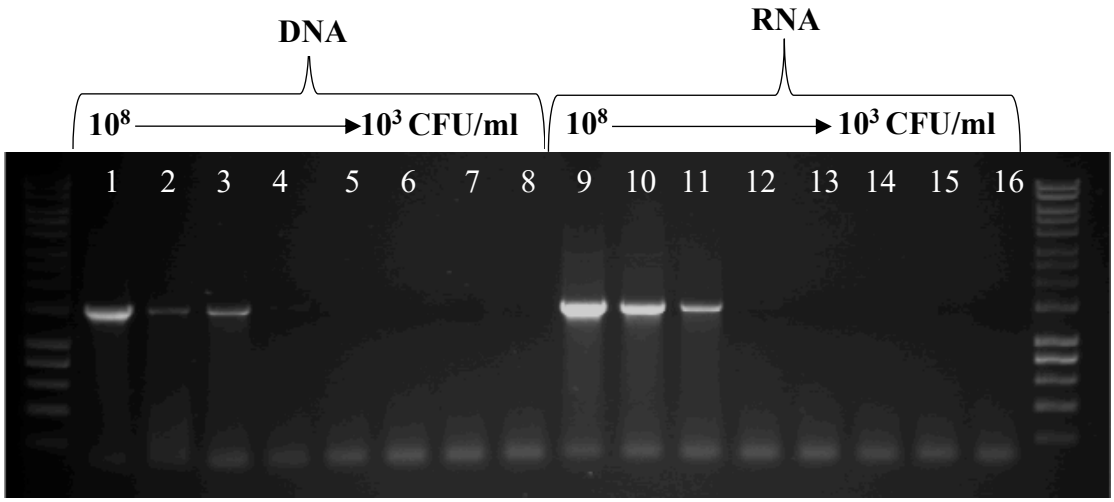


Figure 13: DNA and RNA extracted from artificially contaminated shampoo spiked with different concentrations of *B. cenocepacia* strain 1558. Duplicate serial dilutions of shampoo spiked with *B. cenocepacia* and processed using the filtration method. One set of dilutions followed the RNA extraction protocol and one set of dilutions followed the DNA extraction protocol. Extracted RNA was converted into cDNA. cDNA and DNA was end-point PCR amplified using universal primers (27f and 1492r) and visualised on a 1% agarose gel with Midori green DNA stain using electrophoresis. Lanes 1-8: DNA PCR amplicons, Lanes 9-16: cDNA PCR amplicons. From left to right, the CFU/ml decreases tenfold from 2.25 x 10^8 CFU/ml (lane 1 and 9) to 2.25 x 10^3 CFU/ml (lanes 8 and 16). The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp.

3.**4**.2.1.4.1 Optimization of Amplification and Visualisation of Nucleic Acid: Input Volume in PCR

Increasing the PCR template volume from 2 µl to 5 µl and 10 µl did significantly improve detection sensitivity (Figure 14). There was a 10-fold increase when PCR template concentration was increased from 2 µl to 5 µl. Although detection sensitivity was not increased when PCR template

concentration was increased from 5 µl to 10 µl, crude analysis of gel bands shows brighter and thicker bands at 10 µl.

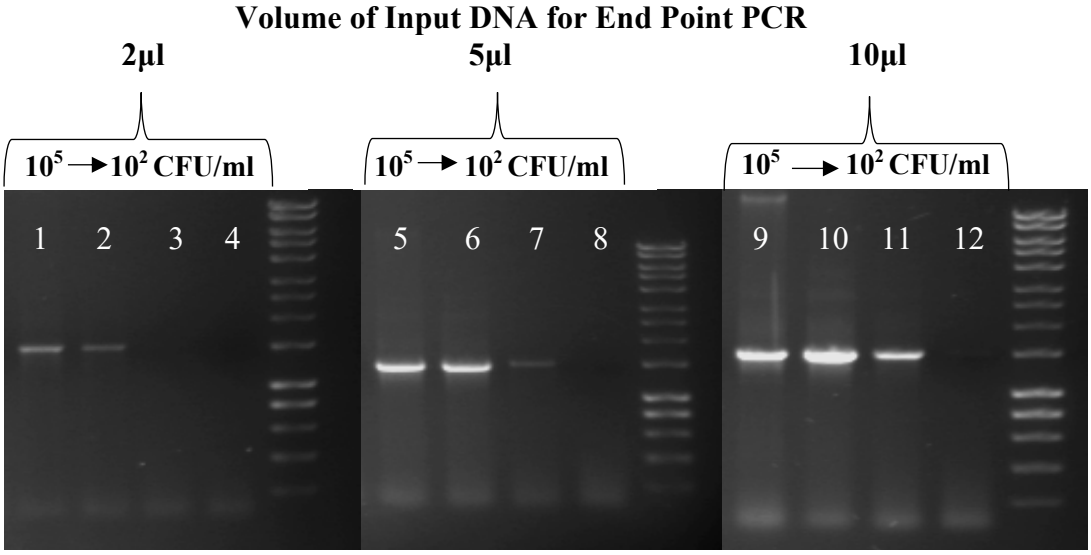


Figure 14: The effect of volume of DNA used in PCR on detection of *B. cenocepacia* strain J2315 spiked into shampoo. DNA was extracted from diluted shampoo spiked with different concentrations of *B. lata* strain 1554 and processed using the filtration method. End point PCR's were performed using universal 16S ribosomal RNA gene primers (27f and 1492r) and 2µl (lanes 1-4) 5µl (lanes 5-8) and 10µl (lanes 9-12) of DNA sample. Amplification products (1465bp) were visualized on a 1% agarose gel with Midori green DNA stain using electrophoresis. From left to right in each shampoo concentration CFU/ml decreases tenfold from 8.36 x 10^5 CFU/ml (lanes 1, 5, 9) to 8.36 x 10^2 CFU/ml (lanes 4, 8, 12). The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp. The negative PCR control of PCR mixtures with primers (27f and 1492r), but with no added cDNA and the positive experiment/PCR control consisted of *B. lata* strain 1554 culture grown overnight and processed as experimental samples (data not shown).

3.4.2.1.4.2 Optimization of Amplification and Visualisation of Nucleic Acid: Use of SYBR Gold Gel Stain

Attempts to increase crude gel electrophoresis band visualisation through use of SYBR gold electrophoresis gel staining, did not increase detection sensitivity but actually decreased sensitivity (Figure 15). As seen in the Figure 15 A, where lanes 1-11 were stained with Midori Green clear visualisation of thick bright bands in lanes 1,7 and 8 can be seen with a faint band in lane 2. However, with additional SYBR gold staining (Figure 15 B), lanes 1,7 and 8 are now significantly reduced in brightness and the faint band in lane 2 has disappeared.

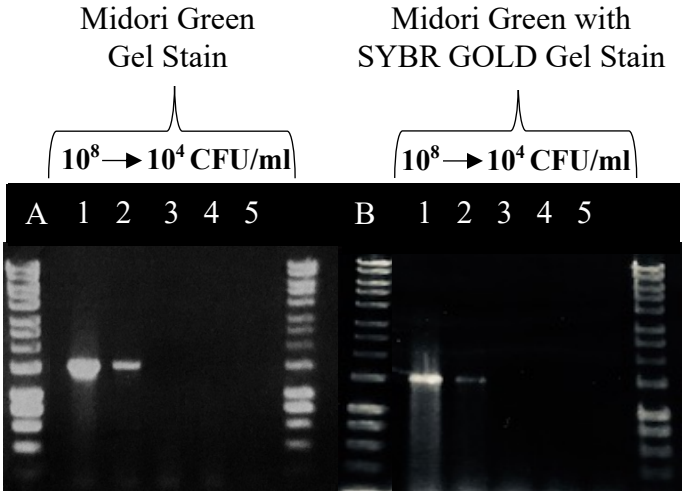


Figure 15: The effect of different electrophoresis gel stains on detection of *B. cenocepacia* strain J2315 spiked into shampoo. DNA was extracted from diluted shampoo spiked with different concentrations of *B. lata* strain 1554 and processed using the filtration method. End point PCR's were performed using universal 16S ribosomal RNA gene primers (27f and 1492r) and 2µl (lanes 1-4) 5µl (lanes 5-8) and 10µl (lanes 9-12) of DNA sample. Amplification products (1465bp) were visualized using gel electrophoresis on a 1% agarose gel with A) Midori Green and B) with SYBR Gold Staining. From left to right in each shampoo concentration CFU/ml decreases tenfold from 3.57 x 10^8 CFU/ml (lanes 1, and 7) to 3.57 x 10^4 CFU/ml (lanes 5 and 11). The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp.

3.4.2.1.4.2 Optimization of Amplification and Visualisation of Nucleic Acid: qPCR

qPCR was performed with gDNA and plasmid standards using universal and *Burkholderia* specific qPCR primers (Table 6). The standard curve for the *Burkholderia* specific primers against both gDNA and plasmid standard produced a qPCR efficiency of <80%. Therefore, qPCR was not deemed a suitable method for reliable quantification of Bcc detection.

3.4.2.2 Narrow Range Detection Methodology

The narrow range detection protocol involved only two steps: sample treatment and nucleic acid amplification and visualisation. Both sections of this protocol underwent optimisation experiments. Due to the crude nature of boil preparation PCR, great variability was seen within results. Therefore, averages of multiple experiments have been given along with a false negative rate, i.e. where tests failed to produce any results.

3.4.2.2.1 Sample Treatment

Shampoo was diluted to 1/1000 in sterile water. Bcc strains were cultured for 18 hours in 3 ml of BSM at 30°C and serially diluted in the shampoo and water to give a concentration gradient from ~1 x 10^8 CFU/ml to ~1 x 10^3 CFU/ml. 1 ml of each dilution was boiled at 95°C for 10 minutes and then centrifuged at 1500 x g for 10 minutes. PCR amplification and amplicon visualisation were then performed as outlined in 3.3.2.2.2. This method achieved an average detection sensitivity of 3.33 x 10^7 CFU/ml (Table 8). However, dilution of shampoo in water was seen to produce some null results with a false negative rate of 2/10 experiments. The lack of observed consistency with water diluents could be caused by inhibitory shampoo substances.

3.**4**.2.2.1.1 Optimisation of Sample Treatment for Boil Prep Methodology: Diluent

In order to investigate if the use of neutralizers like Tween polysorbate 80 increased robustness of the protocol, three concentrations of Tween 80 were examined, 2%, 5% and 10%. In the assessment of optimal diluent type for boil prep PCR, 0.1% Peptone + 2% Tween 80 had the best recovery of all the Peptone and Tween diluents tested (Figure 16). Diluent testing for a broad range molecular method also conferred that Tween 80 increases molecular detection but not at concentrations above 2% where the Tween 80 interferes with molecular processes/equipment.

0.1% Peptone + 2% Tween 80 had an almost tenfold lower average detection sensitivity of 2.34 x 10^8 CFU/ml than compared to water which achieved an average detection sensitivity of 3.33 x 10^7 CFU/ml (Table 8). However, water was seen to produce some null results, whereas 0.1% Peptone + 2% Tween 80 consistently detected bacterial contamination, albeit at high CFU/ml. Therefore 0.1% Peptone + 2% Tween 80 was used as the recovery diluent for boil prep due to its robustness.

Table 8: Averages for bacterial detection sensitivity (CFU/ml) obtained using the boil prep PCR methodology with various shampoo diluents.

	Water	0.1% Peptone + 2% Tween 80	0.1% Peptone + 5% Tween 80	0.1% Peptone + 10% Tween 80
Average Detection Sensitivity (CFU/ml)	3.3×10^7	2.3×10^8	5.3×10^8	3.7×10^8
Standard Deviation	4.6×10^7	4.1×10^8	5.1×10^8	5.5×10^8
Standard Error	1.5×10^7	1.3×10^8	2.1×10^8	3.2×10^8
Range	1.0×10^8	1.0×10^9	1.0×10^9	9.9×10^8
False Negative Rate	2/10	0/10	1/6	0/3

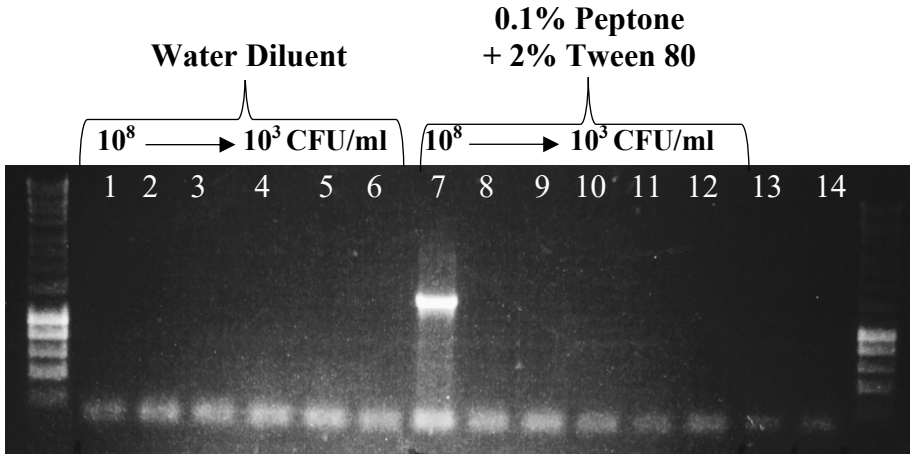


Figure 16: The effect of diluent type on detection of *B. cenocepacia* strain J2315 spiked into shampoo and processed using the boil-prep PCR protocol. Shampoo inoculated with 9.1×10^9 CFU/ml was serially diluted in two different diluents then used in boil prep PCR's using universal primers (27f and 1492r). Amplification products (1465bp) were visualised on a 1% agarose gel with Midori green DNA stain using electrophoresis. Water (lanes 1-6), 0.1% Peptone + 2% Tween 80 (lanes 7-13). The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp. From left to right bacterial cell concentration decreases tenfold from 3.3×10^8 CFU/ml (lanes 1, and 7) to 3.3×10^3 CFU/ml (lanes 6 and 12). The negative experimental control consisted of a shampoo boil prep with water substituted for *B. cenocepacia* (lane 13) and the negative PCR control of PCR mixtures with primers (27f and 1492r), but with no added boil prep lysate (lane 14).

3.4.2.2.1.2 Optimisation of Sample Treatment for Boil Prep Methodology: Lysis Buffer

To examine the effect of an additional lysis buffer treatment of samples prior to the boil prep methodology 100 µl of each dilution was added to 200 µl of lysis buffer solution containing Tris 1 mM, 0.1mM EDTA, 3 µl of 20 mg/ml Proteinase K and 0.5% Tween 20 as described in (Samadi *et al.,* 2007). The samples were incubated for 60 minutes at 35ºC, followed by a 10-minute incubation at 95 °C. PCR amplification and amplicon visualisation were performed as outlined in 3.3.2.2.2. The use of a lysis buffer treatment gave an average bacterial detection of 2.9 x 10^7 CFU/ml which was lower than observed without (Table 9). The use of lysis buffer also consistently produced a result, demonstrating the robustness of the method.

Table 9: Averages for bacterial detection sensitivity (CFU/ml) obtained using the boil prep PCR methodology with a lysis buffer treatment.

	With Lysis Buffer
Average Detection Sensitivity (CFU/ml)	2.9 x 10^7
Standard Deviation	4.0 x 10^7
Standard Error	1.3 x 10^7
Range	9.9 x 10^7
False Negative Rate	0/9

3.4.2.2.2 Nucleic Acid Amplification and Visualisation

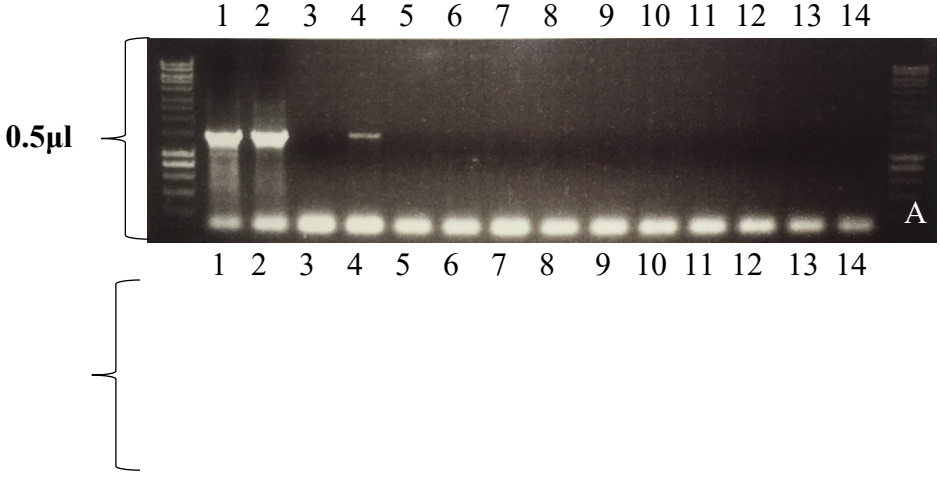
10 µl was used as sample template for end point PCR's with *Burkholderia* Specific primers (See Table 6). Each 50 µl PCR reaction contained 25 µl of Bioline BioMix Red Master Mix (London, UK), 11 µl of nuclease free water, 2 µl of 250 nM forward primer, 2 µl of 250 nM reverse primer and 10 µl of sample. The thermocycler conditions were as follows: 1 cycle of 95 °C for 5 minutes, 35 cycles of 94 °C for 1 minute, 60 °C for 45s and 72 °C for 45s, and 72 °C for 10 minutes. PCR products were visualised using a UV transilluminator, on a 1% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany) using electrophoresis techniques.

3.4.2.2.2.1 Optimisation Amplification and Visualisation of Nucleic Acid for Boil Prep Methodology: Input Volume in PCR

In order to increase the likelihood of low-level DNA amplification, 0.5 µl, 2 µl and 10 µl of sample was added to PCR reactions and bands were visualised and compared as outlined 3.3.2.2.2. There was little difference in the average bacterial detection sensitivity when using 0.5 µl and 2 µl boil prep lysates in PCR (Figure 17). However, adding 10 µl of boiled lysate caused false negatives presumably caused by the increased presence of inhibitory substances (see Table 10).

Table 10: **Averages for bacterial detection sensitivity (CFU/ml) obtained using three volumes of boil prep lysates in end point PCR.**

	0.5 µl	2 µl	10 µl
Average Detection Sensitivity (CFU/ml)	1.4×10^8	1.3×10^8	9.5×10^7
Standard Deviation	2.1×10^8	2.2×10^8	2.2×10^8
Standard Error	4.8×10^7	4.9×10^7	5.2×10^7
Range	1.0×10^9	1.0×10^9	1.0×10^9
False Negative Rate	0/19	0/19	6/19



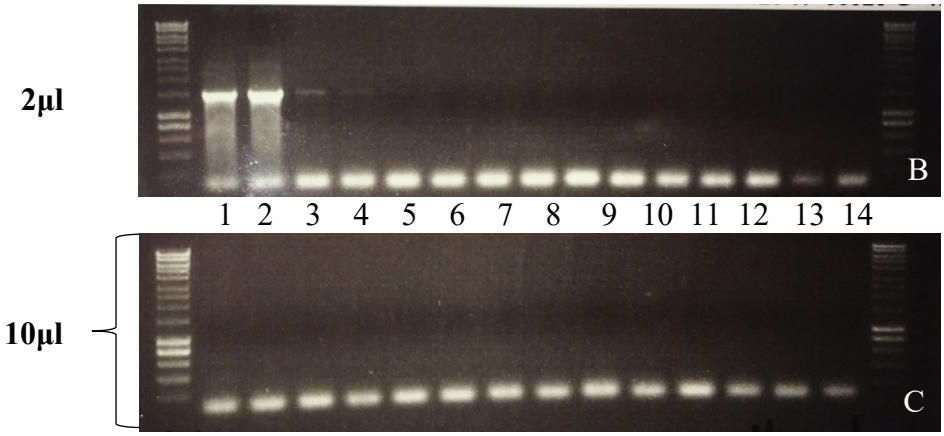


Figure 17: The effect of volume of DNA used in PCR on detection of *B. cenocepacia* strain J2315 spiked into shampoo and processed using the boil-prep PCR protocol. Lysates were added to PCR's in three volumes; 0.5 µl : (Figure A: lanes 1-14), 2 µl : (Figure B: lanes 1-14), 10 µl : (Figure C: lanes 1-14) and amplified using universal primers (27f and 1492r). Amplification products (1465bp) were visualised on a 1% agarose gel with Midori green DNA stain using electrophoresis. The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp. From left to right in each figure, bacterial cell concentration decreases tenfold from 1.9 x 10^9 CFU/ml (lanes 1) to 1.9 x 10^5 CFU/ml (lane 12). The negative experimental control consisted of a shampoo boil prep with water substituted for *B. cenocepacia* (lane 13) and a negative PCR control of PCR mixtures with primers (27f and 1492r), but with no added sample (lane 14).

3.4.2.2.2.1 Optimisation Amplification and Visualisation of Nucleic Acid for Boil Prep Methodology: Lysis buffer and Nested PCR

For the lysis buffer treatment and nested PCR approach the lysis buffer treatment was followed as outlined in 3.3.2.2.1.2 . PCR reactions were set up as previously described with broad range universal primers 27f and 1492r with the extension temperature of 40 °C (Table 6). First round PCR products were visualised on 1% TAE agarose gel with Midori green DNA stain (Nippon Genetics Europe, Dueren, Germany). First round PCR products were serially diluted in sterile water 1/1000 and 10 µl was added to second round PCR reactions with *Burkholderia* specific primers with an extension temperature of 60°C (Table 6). Second round PCR products were visualised as previously described in section 3.3.2.2.2. This was repeated three times. The nested PCR protocol with lysis buffer was highly sensitive (Figure 18). The second round PCR gave an average bacterial detection sensitivity of 4.0 x10^3 CFU/ml with a relatively low standard deviation, standard error and range values (Table 11). It also did not give false negatives within the small sample size tested.

Table 11: Averages for bacterial detection sensitivity (CFU/ml) obtained using the boil prep PCR methodology with a lysis buffer treatment and with a Nested PCR method involving a first-round lysis buffer treatment and universal 16s rRNA primers and second round PCR with *Burkholderia* specific primers.

	With Lysis Buffer and Nested PCR
Average Detection Sensitivity (CFU/ml)	4.0×10^3
Standard Deviation	5.2×10^3
Standard Error	3.0×10^3
Range	9.0×10^3
False Negative Rate	0/3

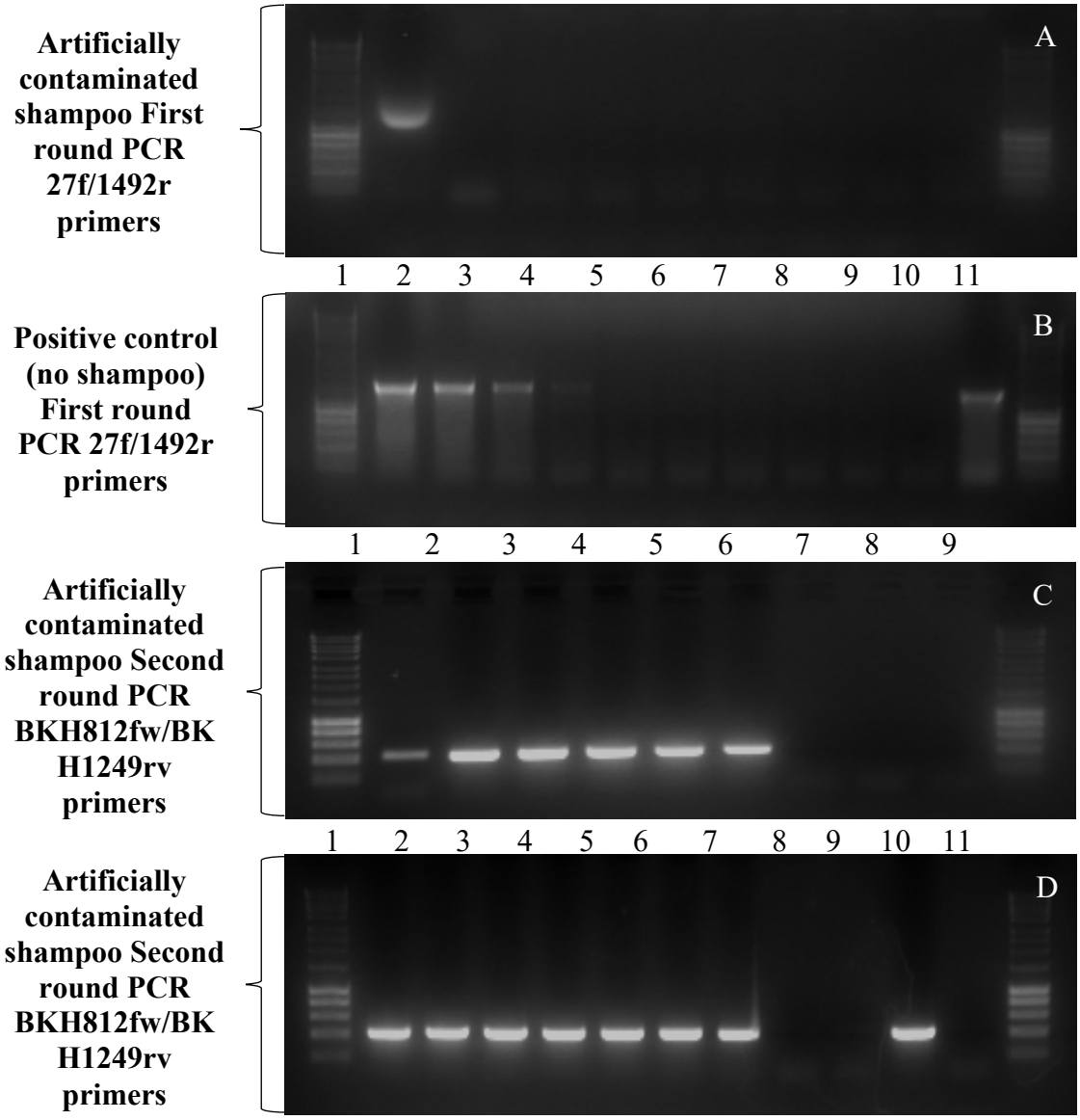


Figure 18: The effect of incorporating a lysis buffer treatment and Nested PCR protocol on detection of B. cenocepacia strain 1558 spiked into shampoo and a water control processed using the boil-prep PCR methodology.

Figure 18: A) and B) First round PCR conducted with universal primers (27f and 1492r) and amplification products (1465bp) were visualised on a 1% agarose gel with Midori green DNA stain using electrophoresis. Lysis buffer treated boil preps using A) shampoo and B) water artificially contaminated with 7.6 x 109 CFU/ml of B. cenocepacia strain 1558 and serially diluted in 0.1% Peptone + 2% Tween 80. The negative PCR control was PCR mixtures with primers but with no added DNA (Fig B, lane 10). The positive PCR control consisted of PCR mixtures with primers and 2 µl of B. cenocepacia strain 1558 DNA (Fig 18 B, lane 11). Figure 18: C) and B) First round PCR diluted 1/1000 and used as template in second round Nested PCR's using Burkholderia specific primers (BKH812 fw and BKH1249 rv) and amplification products (438bp) were visualised on a 3% agarose gel with Midori green DNA stain using electrophoresis. The molecular ladder is Bioline Hyperladder 1 with a size range of 200 - 10,037bp. The negative PCR control was PCR mixtures with primers but with no added DNA (Fig 18 D, lane 11). The positive PCR control consisted of PCR mixtures with primers and 2 µl of B. cenocepacia strain 1558 DNA (Fig 19 B, lane 10).

3.4.3 Assessing Method Utility on Archived Contamination Samples

Unilever provided 17 archived personal care samples. Traditional TVC's were performed to estimate total bioburden (outlined in section 2.4.3). The lysis buffer and nested boil prep-based PCR detection methodology was followed as outlined in section 3.3.4.1 but with the addition of universal primers and *Pseudomonas* specific primers in second round PCR (see table 6).

Total viable counts for the 17 archived contamination samples found only 7 of the 17 still breached the $>10^3$ CFU/ml contamination threshold. 6 products had no detectable viable cells and 4 had a TVC count of less than the 10^3 CFU/ml contamination threshold (Table 12).

Table 12: **Details of Unilever archived contamination samples including Sample Type, Total Viable Count (performed as outlined previously) and Bacterial Strain ID as recorded by Unilever (methods unknown).**

Sample number	Sample Type	Year of Contamination Event	Total Viable Count (CFU/ml)	Bacterial Strain ID
1	Shaving cream	2012	Not detectable	*Nesterenkonia lacusekhoensis*
2	Fabric softener	2006	Not detectable	*Burkholderia cepacia*
3	Fabric softener	2014	8.3×10^2	*Burkholderia lata*
4	Wood cleaner	2013	4.6×10^4	*Burkholderia gladioli*
5	Fabric softener	2013	Not detectable	*Gluconacetobacter sacchari*
6	Shower wash	2006	5.2×10^4	*Burkholderia cepacia*
7	Washing liquid	2012	8.5×10^2	*Unknown*
8	Fabric softener	2008	Not detectable	*Burkholderia cepacia*
9	Crème bath	2002	7.6×10^4	*Pseudomonas putida*
10	Hard surface cleaner	2013	3.4×10^2	*Halomonas desiderata*
11	Shower wash	2007	9.8×10^2	*Burkholderia cepacia*
12	Washing liquid	2011	3.0×10^3	*Burkholderia anthinia*
13	Washing liquid	2008	Not detectable	*Pseudomonas monteilii*
14	Washing liquid	2014	1.4×10^6	*Pseudomonas aeruginosa*
15	Washing liquid	2009	3.9×10^5	*Pseudomonas pseudoalcaligenes*
16	Shower wash	2004	7.2×10^5	*Burkholderia tropicalis*
17	Floor Cleaner	2013	Not detectable	*Pseudomonas aeruginosa*

Electrophoresis visualization of bands on the agarose gel for first round PCR using universal primers displayed a linear relationship for amplified DNA and serial dilutions, with the highest dilutions having increased brightness and thickness of PCR bands (see Figure 19A and 19B).

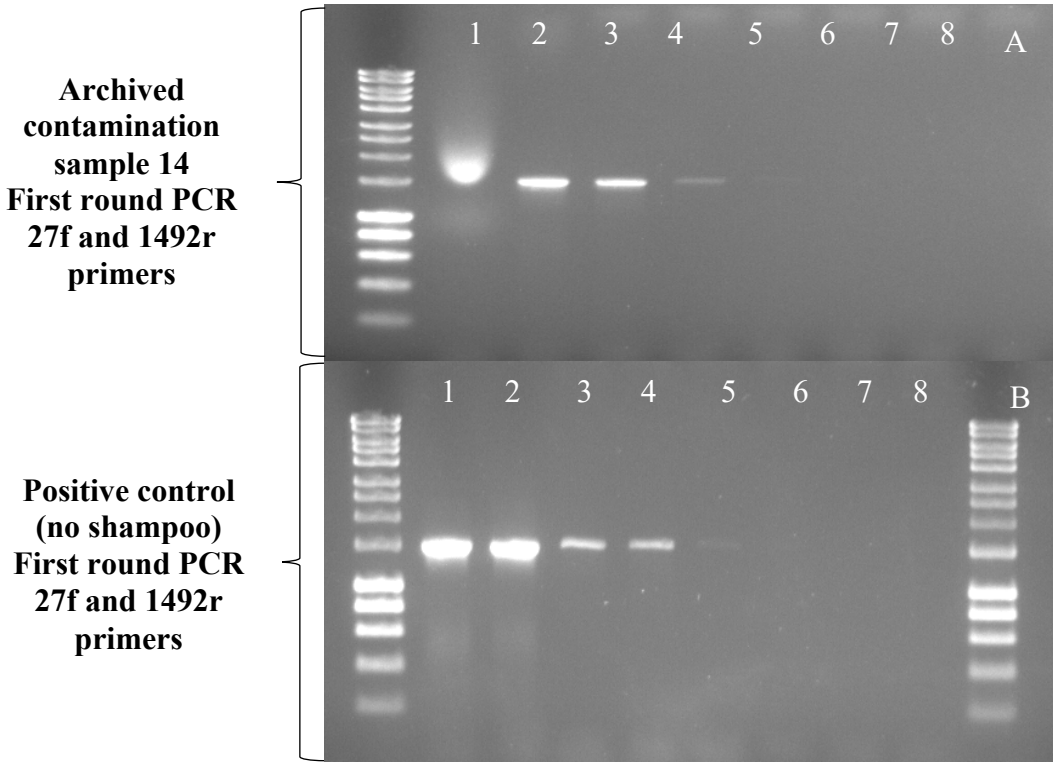


Figure 19: A) First round PCR of lysis buffer treated boil preps using archived contamination samples serially diluted in 0.1% Peptone + 2% Tween 80. Lanes 1-8: Sample 14.

Figure 19: B) First round PCR of lysis buffer treated boil prep positive control (no shampoo) using 8.2 x 10^9 CFU/ml of *B. cenocepacia* strain 1558 serially diluted in 0.1% Peptone + 2% Tween 80. First round PCR conducted with universal primers (27f and 1492r) and amplification products (1465bp) were visualised on a 1% agarose gel with Midori green DNA stain using electrophoresis.

Unlike first round PCR, electrophoresis visualization of bands on the agarose gel for the second round PCR did not display a relationship for amplified DNA and serial dilutions, (see figure 20A, 20B and 20C).

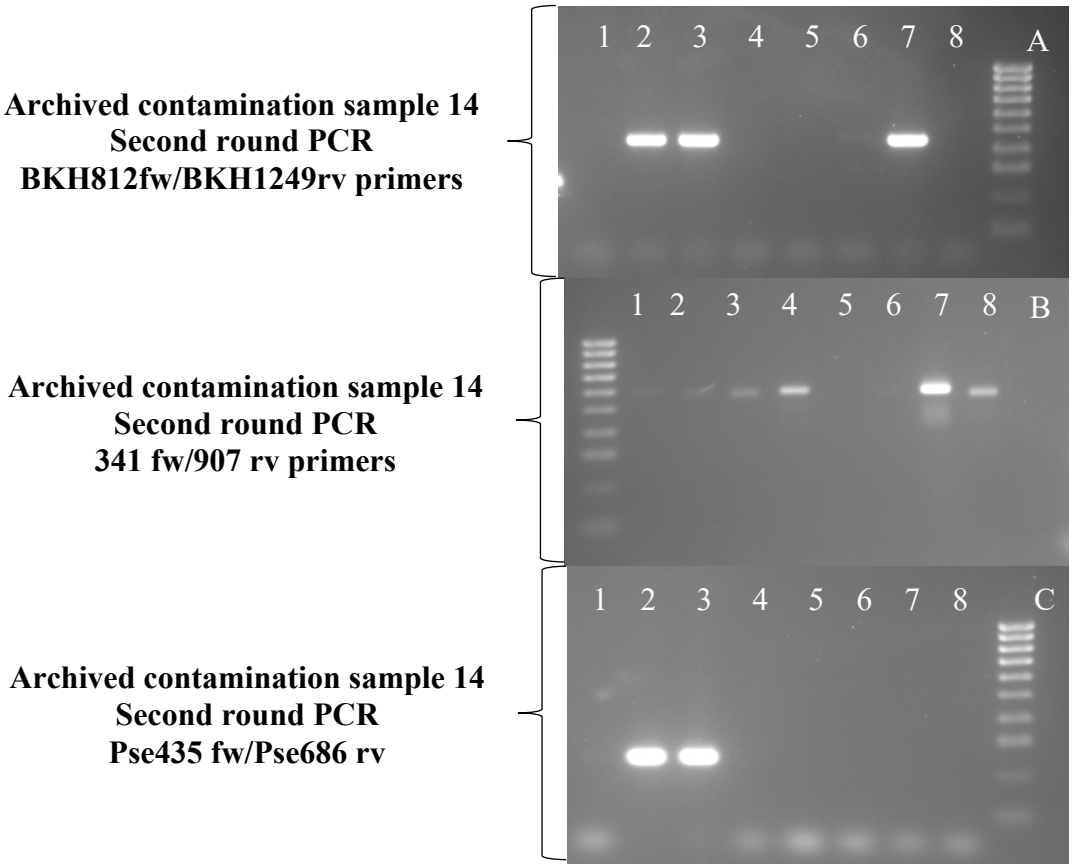


Figure 20: A) Second round PCR of lysis buffer treated boil preps using archived contamination samples serially diluted in 0.1% Peptone + 2% Tween 80. First round PCR products were diluted 1/1000 and used as template in second round Nested PCR's with; *Burkholderia* specific primers (BKH812 fw and BKH1249 rv) producing amplification products at 438bp (Figure 20 A), universal primers (341 fw and 907 rv) producing amplification products at 566bp (Figure 20 B) and *Pseudomonas* specific primers (Pse435 fw and Pse686 rv) producing amplification products at 251bp (Figure 20 C). Amplification products were visualised on a 3% agarose gel with Midori green DNA stain using electrophoresis. The molecular ladder is Bioline Hyperladder 4 with a size range of 100 bp to 1013 bp. The negative PCR control was PCR mixtures with primers but with no added DNA and the positive PCR control consisted of PCR mixtures with primers and 2 µl of *B. cenocepacia* strain 1558 DNA (data not shown).

13 of the 17 first round PCR's using universal primers amplified DNA (See Table 14). The 4 products which did not produce any first round PCR results (2, 5, 8, 17), also did not produce a TVC (See Table 13). 2 products that had no detectable viable cells (13 and 1) and 4 products that had a TVC count of less than the 10^3 CFU/ml contamination threshold (3, 7, 10 and 11) produced a first round PCR signal. There was a weak correlation for TVC count and number of first round PCR bands visualized, with high TVC's producing more PCR bands (Figure 21).

17 of the 17 second round PCR's using universal primers amplified DNA (See Table 14). There was no relationship between the count of visible PCR bands from gel electrophoresis imaging of first round PCR using universal primers and count of visible PCR bands from gel electrophoresis imaging of second round PCR using universal primers, i.e. no/few first round bands did not correspond with no/few second-round bands (see Figure 22).

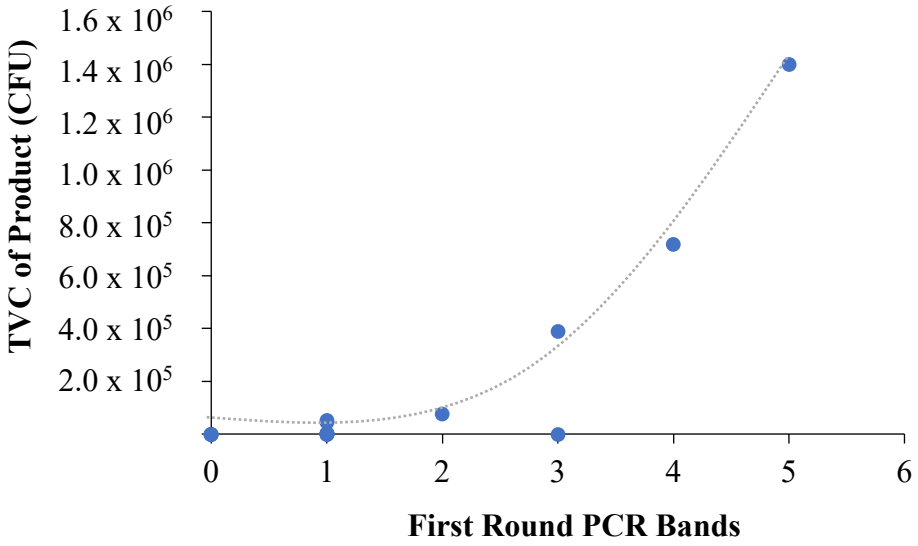


Figure 21: The relationship between Total Viable Count (CFU/ml) and visualisation of PCR bands by gel electrophoresis after lysis buffer boil preparation and first round PCR using universal 16s rRNA primers 27f/1492r.

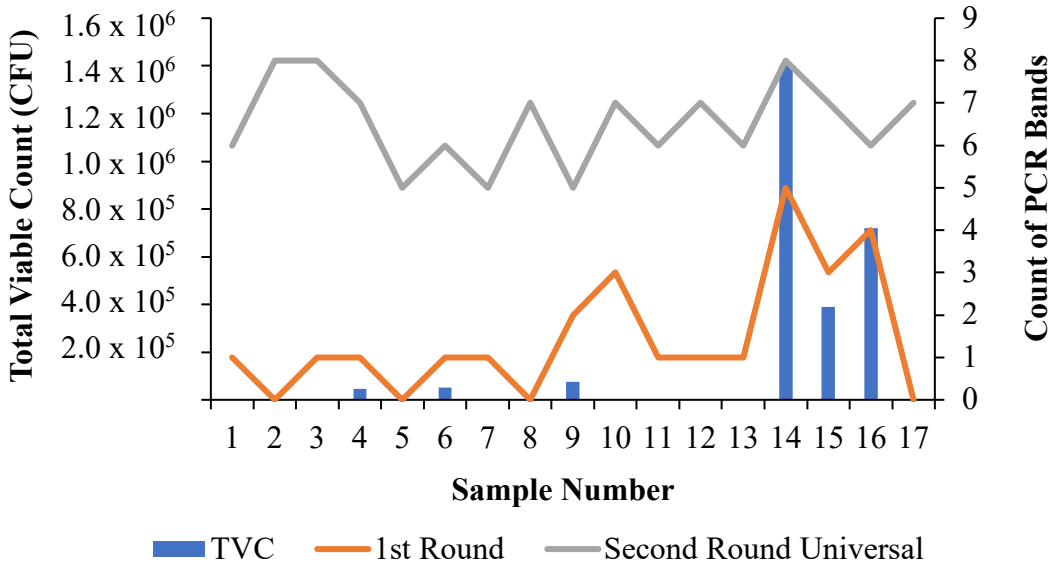


Figure 22: The relationship between Total Viable Count and visualisation of PCR bands by gel electrophoresis after in lysis buffer boil preparation and Nested PCR detection methods. Nested PCR employed first round universal 16s rRNA primers 27f/1492r and second round universal 16s rRNA primers 341f/907r.

Both sets of specific primers had poor specificity, although the *Pseudomonas* specific primers did perform better than the *Burkholderia* specific primers (see Table 14). For the five products contaminated with *Pseudomonas* strains, the average count of visible PCR bands produced using *Pseudomonas* specific primers and visualized using gel electrophoresis imaging was 6, however it did also cause an average of 4 band for the eight products contaminated with *Burkholderia* species and an average of 2 bands for the three non-*Burkholderia* or *Pseudomonas* genus contaminants. For the eight products contaminated with *Burkholderia* strains, the average count of visible PCR bands produced using *Burkholderia* specific primers and visualized using gel electrophoresis imaging was 2, and actually produced a higher average of 4 bands in the five products with *Pseudomonas* species contamination and an average of 3 bands for the three other non-*Burkholderia* or *Pseudomonas* genus contaminants.

Table 13: **Averages for PCR bands obtained using the boil prep PCR methodology with lysis buffer treatment and nested PCR method. First round PCR was conducted with universal 16s rRNA primers and second round PCR was conducted with universal primers 16s rRNA primers, *Pseudomonas* Specific Primers and *Burkholderia* Specific Primers.**

		First Round PCR	Second Round PCR		
Sample Number	Sample Species ID	Universal Primers	Universal Primers	Pseudomo-nas Specific Primers	Burkhold-eria Specific Primers
1	*Nesterenkonia lacusekhoensis*	1	6	3	1
2	*Burkholderia cepacia*	0	8	5	3
3	*Burkholderia lata*	1	8	3	1
4	*Burkholderia gladioli*	1	7	6	2
5	*Gluconacetobacter sacchari*	0	5	1	0
6	*Burkholderia cepacia*	1	6	4	3
7	*Unknown*	1	5	3	0
8	*Burkholderia cepacia*	0	7	0	3
9	*Pseudomonas putida*	2	5	5	3
10	*Halomonas desiderata*	3	7	3	4
11	*Burkholderia cepacia*	1	6	3	2
12	*Burkholderia anthinia*	1	7	5	1
13	*Pseudomonas monteilii*	1	6	2	1
14	*Pseudomonas aeruginosa*	5	8	8	3
15	*Pseudomonas pseudoalcaligenes*	3	7	6	2
16	*Burkholderia tropicalis*	4	6	2	0
17	*Pseudomonas aeruginosa*	0	7	6	6

3.5 Discussion

3.5.1 Overcoming the Inhibitory Environment of Personal Care Formulations

Under current harmonised pharmacopeia, a high nutrient broad range media such as TSA should be used for the "Total Microbial Enumeration" test (European Pharmacopoeia, 2016a). However, there is a concern that the recovery of sub-lethally damaged, stressed or environmentally adapted bacteria is not achieved with broad range media as used in growth-based culture techniques. Bcc are highly adaptable organisms and can easily acclimatise to environments with limited nutrition e.g. soil and water environments. However, acclimatized low-nutrition Bcc strains would then struggle to grow in high nutrient media and this could exert nutrient stress effects. Stressed cells exhibit increased cell membrane permeability leading to leaking of intracellular metabolites such as amino acids, potassium, proteins, as well as a causing a decrease in enzyme activity (Lancette, 2016). The decreased activity of the enzyme catalase is seen to cause the toxic accumulation of hydrogen peroxide which results in oxidative stress and cell damage/death (Leist *et al*., 1996). Therefore, low nutrient media, as well as the addition of the hydrogen peroxide degrading enzyme catalase was used to increase the enumeration of sub-lethally damaged cells from shampoo. However, no media or catalase combination was seen to increase Bcc enumeration from artificially contaminated personal care product experiments (Table 7). The lack of preference for high or low nutrient media is supported by a study conducted in 2014 investigating media preference of cold-water acclimated Bcc organisms which found that recovery on both nutrient rich broth media (Tryptic soy broth with lecithin plus Tween and R2A broth) and low nutrient media (10% TSB with lecithin and Tween and 10% R2A broth) was similar after 48 hours of incubation at 30-35°C (Barlasov *et al.,* 2014).

Another hypothesis for the lack of media preference could be that as healthy Bcc cells were inoculated into shampoo, they were not sub-lethally damaged/acclimated to the nutrient poor environment shampoo. Attempts to circumvent this by "growing" acclimated Bcc cells in shampoo, both with and without preservatives and with different Bcc genomovars was not successful.

3.5.2 The Use of the Neutralising Compound Tween Polysorbate 80 to Counteract the Inhibitory Effect of Shampoo and its Preservatives

Preservatives are used to suppress microbial growth or kill microorganisms which enter products through raw material, the manufacturing process or even in use. In the examination of pharmaceutical products, neutralising substances are added to the culture broth and media in order to counteract the presence of anti-microbial preservatives in formulations. This is demonstrated experimentally whereby the addition of 2% Tween 80 to 0.1% peptone produced a 10-fold increase in mean total viable counts (Table 7). In order to determine if the addition of neutralisers, such as Tween 80, enhanced molecular methods for bacterial detection both broad range methodologies (centrifugation and filtration) and the narrow range detection methodology (boil prep PCR) were performed in 0.1% peptone solution and 0.1% peptone + 2% Tween 80 solution. For all molecular detection methodologies, (broad range and narrow range) the use of 2% Tween 80 increased PCR sensitivity of Bcc amplification from shampoo (Figure 8). This supports existing research that non-ionic detergents such as Polysorbate 80 have been seen to counteract the effect of trace detergents, which may have an inhibitory effect on PCR amplification (Konat *et al.,* 1994).

Harmonised EP and USP regulations allow the use of Tween polysorbate 80 concentration of up to 10%. Therefore, increasing Tween polysorbate 80 concentrations used in the diluents for shampoo dilution was investigated. For broad range methodologies increasing the concentration of Tween 80 above 2% resulted in a reduction in detection sensitivity (Figure 9). This is presumably caused by Tween 80 emulsion/surfactant properties, which at high concentrations could stop the separation of inhibitory shampoo and bacterial cells and also prevent pellet formation during centrifugation steps. For narrow range detection methodologies, there was little difference between increasing Tween 80 concentrations, although 2% Tween 80 did achieve the highest detection sensitivity out of all the Tween 80 diluents tested (Table 8).

In order to determine if dilution effects were sufficient to negate the presence of preservatives in the shampoo, two concentration gradients of shampoo and bacterial cell count were tested. A linear relationship was found with the lower concentration

gradients of shampoo expressing better detection sensitivity, even at lower bacterial cell concentrations (Figure 10). However, if we consider a real-world contaminated product protocol, these two variables would not be separated, shampoo concentration and bacterial cell concentrations would both be reduced with serial dilution. Therefore, whilst serial dilution is a successful solution to diluting out inhibitory shampoo, the dilution of bacterial cell count significantly reduces detection capabilities. This is seen with both broad range and narrow range detection protocols where serial dilution of shampoo and bacterial cell count in tandem results in poor detection sensitivity (Figures 8-9, 11-18).

3.5.3 Evaluation of Nucleic Acid as a Target for Detection

It was hypothesised that RNA would be superior target for detection than DNA due to its ability to indicate the presence of viable cells and the greater abundance of ribosomal RNA present in the cell (Steward *et al.,* 2005). However, sub-lethally damaged, stressed, VBNC cells have a reduced metabolic activity, decreasing mRNA content and making its cellular abundance comparable to DNA (Hammes *et al.,* 2010). Therefore, simultaneous extraction of DNA and RNA from Bcc cultures and shampoo spiked with Bcc was investigated. Extraction of DNA and RNA from Bcc cultures produced almost 50% higher yield of RNA than DNA (Figure 4). However, efficient-high yield extraction of ribosomal RNA from bacterial culture requires a 12-hour incubation with an RNA stabilisation agent, and the omission or truncation of this step results in a significantly decreased yield which is comparable to DNA yields from the same bacterial culture (Figure 3). This is problematic as discussions with Unilever, the CASE partner, highlighted the need for a quick assay result (within a 7-hour manufacturing shift). Simultaneous extraction of DNA or RNA from artificially contaminated shampoo using commercially available kits resulted in a similar yield and comparable detection efficiency, therefore DNA was used as the nucleic acid target for detection.

3.5.4 Molecular Methodologies for Sensitive, Rapid Bacterial Detection

Initial results of EMA-PCR did not discriminate viable and non-viable cells well and resulted in both the underrepresentation of viable cells and the overrepresentation of non-viable cells (Figure 5/6). Over-representation of non-viable cells has also been reported by various studies attempting to employ viability PCR methods (Fittipaldi

et al., 2011, 2012; Soejima *et al.,* 2011; Kralik *et al.,* 2010; Bae and Wuertz, 2009; Kobayashi *et al.,* 2009; Flekna *et al.,* 2007; Nocker and Camper, 2006). A published study employing a similar EMA protocol on *Staphylococcus aureus* and *Staphylococcus epidermidis* viable and heat treated non-viable cells found that regardless of numerous optimisation experiments, EMA-PCR under-represented viable cells and did not significantly affect non-viable cell DNA amplification (Chai *et al.,* 2009).

The greatest disadvantage of EMA, is penetration of both intact and compromised bacterial cell membranes, which require careful validation of the reaction conditions (dye concentration, dye incubation conditions, the chosen light source and exposure time) and the sample conditions (the ratio of viable and non-viable cells, the presence of inhibitory substances in the sample) as well as the PCR conditions (gene length and salt concentration) (Fittipaldi *et al.,* 2012). Due to poor initial results attempting to remove non-viable cell signal (Figure 7) further validation was not conducted. In order for EMA PCR to be employed by Unilever, the CASE partner, in-depth validation would have been required with multiple strains (~30) across a diverse range of sample matrices (~60) as EMA PCR results vary sample to sample and strain to strain, however this magnitude of work was not in the scope of this PhD. Furthermore, EMA uptake by viable and non-viable cells is greatly affected by strain cellular permeability. Although EMA PCR methodologies were never used with artificially contaminated personal care products, damaged and stressed cells, such as industrial contaminants have highly permeable membranes, which would almost certainly result in EMA uptake by viable cells resulting in the underestimation of true viability.

There are many practical and theoretical limitations to the application of this method to “real world” samples. Matrix choice is important for EMA PCR, and even low complexity environmental samples such as water have resulted in poor estimates viable micro-organisms (Varma *et al.,* 2009) . Therefore, the utility of this method for the complex chemical matrices of personal care products would not be advised.

3.5.5 Developing a Broad Range Detection Methodology and Optimising for Increased Sensitivity

Centrifugation protocol which employed the centrifugation of shampoo dilutions only achieved detection of significant bacterial contamination of 2.20 x 10^6 CFU/ml and above (Figure 11). This method also required technical skill as there was a lack of solid pellet formation due to the presence of surfactants and attempts to circumvent this by increasing centrifugation speeds did not improve this. Analysis of the spectral profiles of nucleic acid extracted by centrifugation method using the Nanodrop Spectrophotometer demonstrated that this method did not produce "pure" DNA, with shifted troughs indicative of sample contamination, making it unsuitable for further downstream molecular processes (e.g. qPCR). Furthermore, centrifugation also requires the expensive purchase ultracentrifuge equipment.

Filtration protocol which employed filtration of shampoo dilutions could also only detect contamination in shampoo at bacterial loads of 3.57 x 10^6 CFU/ml and above (Figure 12). The filtration protocol achieved the same log recovery as the centrifugation protocol with good quality nucleic acid (uncontaminated with shampoo) and using filtration equipment which is easy to use and common in QC laboratories.

3.5.6 Lysis Buffer Boil Preps with Nested PCR and Blind Testing on Archived Contamination Samples

Of the small sample of academic research investigating PCR detection of pharmaceutical non sterile products and personal care products (a total of 12 papers) only one has reported a low detection sensitivity from artificially contaminated samples without a pre-enrichment step (Samadi *et al.,* 2007). A paper by Samedi *et al.,* (2007) detected 10^2 CFU/ml contamination from a non-sterile lotion, without enrichment in 1-2 hours by using enzymatic cell degrading treatments followed by a boil-prep. (Samadi *et al.,* 2007) used a lysis buffer containing proteinase K, one of the most commonly used enzymes for the disruption of bacterial cells. Proteinase K is a broad-spectrum serine protease, isolated from the fungus *Tritirachium album* (Moore *et al.,* 1999). The method employed by this study utilized the direct use of crude lysates from Proteinase K bacterial lysis and boil-prep treatments of artificially contaminated shampoo, with nested PCR techniques to provide a detection

sensitivity of $>10^3$ CFU/ml, the threshold for product contamination. This was achieved in four hours, within the "7-hour shift" time frame.

Application of this lysis buffer boil prep with nested PCR methodology on real world archived contamination samples was highly effective, able to identify contamination in all samples (Figure 18). Where TVC counts of products revealed no detectable cells, DNA was amplified using this technique (Figure 23). It is unclear if PCR successfully identified viable cells that standard culture techniques missed or if PCR's amplified non-viable cells present in the sample.. The utility of this method is very narrow as *Pseudomonas* species specific and *Burkholderia* species specific second round primers had poor sensitivity and specificity, and therefore could not be used for reliable identification (Table 14). *In-silico* analysis of the *Burkholderia* specific 16s rRNA primers used in this study showed between a 78% and 100% compatibility with the 16s rRNA sequences of 55 *Burkholderia* type strains available on the RDP database in 2010 (Bergmark *et al*., 2012). However, this study showed a low compatibility with *Burkholderia* species used in this study, as shown by poor specificity and sensitivity in lysis buffer boil preps with nested PCR on archived contamination samples and its failed use in qPCR experimentations. Several other studies have demonstrated the use of 16s rRNA specific primers is not advised for the reliable detection and distinction of closely related species such as *Pseudomonas* and *Burkholderia*. Current practices for molecular distinction of *Burkholderia* species is based on recA gene analysis or MLST (Coenye and Mahenthiralingam, 2014) . However, with the use of broad range 16s rRNA primers, this lysis buffer boil preps with nested PCR shows a promising utility in confirming bacterial contamination in the "7hour shift" time frame.

3.6 Conclusions

The application of PCR based molecular detection methodologies for bacterial contamination in personal care products is complex. The two main issues that affect its routine use as QC microbiology tool is: the inhibition of amplification by substances found in the complex chemical formulations and the inability to distinguish between viable and non-viable micro-organisms. Despite the challenges, a method was developed utilising lysis buffer boil preps with nested PCR which could provide a quick, sensitive and fast method for confirming spoilage in personal care products.

Chapter 4: Adaptive Bacterial Resistance to Preservatives

4.1 Introduction

4.1.1 Current Issues with Product Preservation

There is no requirement for manufacturers to disclose and publish incidences of product contamination. Inference of rises in microbiological contamination can be gained from product recall data by reporting governing bodies. For the EU this is Safety Gate (formerly the RAPEX: the rapid alert system for non-food consumer products) (European Commission, 2019) and for the US the Food and Drug Administration (FDA, 2020). Data from both the EU and US suggest microbial contamination of non-food industrial product contamination is on the rise. According to data from EU Safety Gate non-food product recalls from 2006 to 2016, reports and recalls involving microbial contaminants for non-food consumer products showed a year on year increase, from 14 reports and 9 recalls in 2006 to 44 recalls and 32 recalls in 2016 (Cunningham-Oakes *et al*., 2020). This is mirrored in microbiological contamination recalls from FDA reported data, whereby recalls between 2004 and 2011 had doubled (Sutton and Jimenez, 2012).

The development of bacterial resistance to preservatives and the subsequent contamination of preserved product by pathogenic bacteria can endanger human health as well as causing serious economic losses for manufacturers (Patton, 2018). For example, in May 2018, Bcc contaminated cleansing wash resulted in a multistate outbreak and caused serious infection in 15 hospitalized patients (FDA, 2018). This has led to great concern in the manufacturing industry, with experts warning such as the words of Phil Hindley the Head of Global Marketing and Preservation for Lonza; “Microbial contamination caused by species like Burkholderia cepacia in consumer products is a key public health issue,” (Lonza, 2018).

4.1.2 Moving Towards the Future: Eco-Preservatives

Preservatives are essential to ensure the sterility and stability of daily use home and personal care products. In order to fight adaptive resistance development to preservatives used in home and personal care industry, formulations are moving

towards natural preservation strategies (Reisch, 2016). Nature identical preservatives offer an alternative to conventional synthetic preservation systems. Various nature identical preservatives are already approved for use and are routinely used in the preservation of foods, medicines and cosmetics. However, their utilisation has been limited as it is believed that in-use concentrations are much higher than synthetic preservatives, making nature identical preservatives less cost effective. It is believed that nature identical agents are not as broad spectrum as synthetic preservatives and offer limited anti-microbial efficacy against gram-negative species (Browne *et al*., 2012). Understanding the relative microbial efficacy of nature identical preservatives against common personal care contaminants such as the Bcc is essential to re-purpose these agents for personal care preservation.

4.1.3 Bacterial Adaptive Resistance

There are three potential mechanisms of bacterial antimicrobial resistance: intrinsic, acquired or adaptive resistance mechanisms. Intrinsic resistance concerns an organism's innate structural or function characteristics which affords greater resistance to antimicrobial substances (Gang and Jie, 2016). Acquired resistance involves a gained genomic mutation or element (plasmid and transposon) which confers increased antimicrobial resistance (Rizi *et al*., 2018). Most of the research pertaining to resistance development has concerned intrinsic and acquired resistance as these are stable and present regardless of environmental conditions. Defining adaptive resistance is difficult as it may lead the induction of acquired and intrinsic resistance mechanisms (i.e. gene expression and gene mutations) which confer stable and broad physiological and antimicrobial resistance traits (Russell, 2004). At present adaptive resistance is considered "a temporary increase in the ability of a bacterium to survive an antibiotic insult due to alterations in gene and/or protein expression as a result of exposure to an environmental trigger" (Lim *et al*., 2012).

Adaptive resistance was previously considered transient and not involved in long-term resistance phenotypes. However, some studies have demonstrated stable adaptive resistance which does not return to wild type when the antimicrobial is removed (George and Levy, 1983). The mechanisms of adaptive resistance emergence and stability are poorly understood. Testing the adaptive resistance

capabilities through gradually increasing amounts of antimicrobial has been described as early as 1983 (George and Levy, 1983).

Whether high dose/single exposure or sub lethal concentrations/prolonged strep wise training methods produce similar adaptive resistance results is highly disputed (Wales and Davies, 2015). It was previously thought that adaptive resistance can be acquired in a short period. However, dose and length of antimicrobial exposure are considered to be important factors in adaptive resistance levels and stability (Fernández *et al*., 2011). A recent paper suggests there are two types of adaptive resistance: fast and transient mechanisms (FTM) and slow and stable mechanisms (SSM) (Motta and Aldana, 2016). FTM is characterised by a fast timescale, where small epigenetic changes lead to transient phenotypic resistance, often at a cost to population growth. Due to growth arrest associated with resistance mechanisms, without the selective pressure of the antimicrobial, resistance is reversed. SSM is characterised by a longer timescale, whereby compensatory mechanisms are developed for growth handicaps associated with resistance, and therefor confer stable resistance mechanisms. SSM may also be associated with genetic changes also associated with long term resistance.

Biocides are used throughout the industrial manufacturing environment as preservatives of raw materials and disinfection agents for sanitation of plant surfaces and machinery. In order to maintain efficacy these antimicrobial substances must be used at lethal or inhibitory concentrations (Wales and Davies, 2015). However, preservatives are used at low concentrations due to sensitisation and toxicity risks which increases the likelihood of sub-inhibitory preservation. Sub-inhibitory concentrations of product preservative may occur from degradation by contaminating micro-organisms and formulation interference on preservative activity (Hugo, 1991).

Biocides are not readily biodegradable and environmental biocide concentration gradients may occur as a result of improper cleaning or removal of residue from production lines. Gradients of biocide in manufacturing production lines equipment are of particular concern these can also harbour bacterial biofilms, which themselves confer increased biocide resistance through decreased penetration ability (Orth *et al*.,

2006; Orth, 1985). The environment therefore acts as a long-lasting reservoir for biocides and preservatives and select for resistant microbial populations. Environmental susceptibility to biocides has been seen in various agricultural and industrial settings, where biocides are commonly used as environmental sanitisers in the food chain and in industrial manufacturing plants (Wales and Davies, 2015).

This study will investigate the changes in MIC values before and after response to sub-inhibitory concentrations of preservatives and the potential to induce adaptive resistance response. Whilst biocides and antibiotics are intended to be bactericidal, preservatives are intended to be bacteriostatic. Personal care products must demonstrate an absence of certain pathogenic organisms and total microbiological counts within levels but are not intended to be sterile. Minimum Bactericidal Concentrations (MBC) were not conducted in this study. However, increases in MIC would indicate a lower bactericidal activity. Furthermore, increases in MIC sufficiently demonstrate the microbiological definition of resistance, which is considered to occur when antimicrobial concentrations no longer exert bactericidal effect at concentrations previously see in strains of the same organism (Russell, 2003).

4.2 Aims

The aims of this chapter were as follows:

1. To determine the preservative susceptibility of a collection of 47 *Burkholderia* strains isolated from clinical and industrial environments against synthetic and nature identical preservative agents using minimum inhibitory concentration methodologies.

2. To investigate the potential for Bcc to develop adaptive resistance to natural preservative agents in comparison to synthetic preservative agents, via the progressive subculture of *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318 in/on media containing increasing sub-inhibitory concentrations

3. To examine the potential to induce adaptive resistance of *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318 to preservative agents using low concentrations and slow timeframes compared to high concentrations and fast timeframes.

4. To examine the stability (i.e. the persistence of preservative resistance upon removal of the preservative agent), of preservative adaptive resistant Bcc strains via the sub-culture of adapted derivatives in the absence of preservative, and re-testing of the minimum inhibitory concentration (MIC)

4.3 Methods

4.3.1 Preservative Susceptibility Testing of a Panel of *Burkholderia* strains to Nature identical and Synthetic Preservatives Systems

All methods were derived from Rushton *et al*., 2013.

4.3.1.1 Creation of a Bacterial Master Plate

40 environmental *Burkholderia* strains were cultured for 18 hours in 3 ml of BSM at 30°C in 28 ml sterile universals and shaken at 150 rpm. 7 clinical Bcc strains originally isolated from Bcc sputum samples, *B. cenocepacia* strain J2315, *B. multivirons* strain C5393, *B. ambifaria* strain BC284, *B. dolosa* strain BCC232 and three unknown designation Bcc strains (BCC F655, BCC F651, BCC F768), were cultured for 18 hours in 3 ml of BSM at 32 °C in 28 ml sterile universals and shaken at 150 rpm. Cultures were adjusted to give to approximately $1x10^8$ CFU/ml. Each culture was then frozen in a 96 well Masterblock (Grenier Bio-one, UK) with 8% v/v of DMSO, and sealed using a capmat lid (Grenier Bio-one, UK). For revival of the *Burkholderia* strain collection, the -80 °C masterblock was defrosted on ice for approximately 1 hr and using a multipoint inoculator 1.5 µl of each strain was aliquioted into a 96 flat bottom well plate (Grenier Bio-one, UK) containing 200 µl of Basal Salt Broth (BSM). This plate was then incubated static, at 30 °C for 18 hours and turbidity measurements were red at the start and end of the incubation period by the OMEGA Flurostar microplate reader (BMG Labtech, Germany). When revived by this method strains reached a consistent OD and viable counts between ~$1x10^8$ and ~$1x10^9$ CFU/ml.

4.3.1.2 MIC Determination by Agar Dilution Assay

An agar dilution method was used to investigate the susceptibility of the *Burkholderia* strain panel against the bacteriacides. After autoclaving BSM agar was cooled to 50°C and preservative stock was added to 50 ml of agar to achieve bactericidal concentrations ranges of: benzoic acid 0 – 0.5% w/v, salicylic acid 0 – 0.5% w/v, DMDMH 0 – 0.3% v/v, methyl paraben 0 – 0.4% v/v, benzisolthiazolinone 0 – 0.2% v/v, and methylisothiazoline-chloromethylisothiazolinone 0 – 0.0015% v/v. Agar was poured into vented 120mm

square petri dishes (Grenier Bio-one, UK) and allowed to dry for approximately 24 hours before use. For acid preservatives the pH of the agar was adjusted to pH 5 to avoid a reduction in activity. Viability and growth of Bcc strains on pH 5 media and pH 7 media was compared using five strains representing the most common strain types in the collection, and no effect was observed.

The bacterial masterplate was revived as previously described and used to inoculate pH 7 or pH 5 broth plates. A Boekel Scientific 48 Pin Replicator (Philadelphia, USA) was used to place 1 µl culture onto the surface on the agar plates in duplicate. The replicator was washed with sterile PBS and flame sterilised using 100% ethanol between each inoculation to prevent preservative and bacterial carry over. To prevent cross contamination of different preservatives the replicator was cleaned with 5% v/v Trigene detergent and rinsed thoroughly with sterile water, and the flaming ethanol was changed between different preservative types. Control plates containing no preservative were inoculated with the bacterial maser plate after each set of preservative plates were inoculated. Plates were incubated at 30°C for 24 hours. The MIC was defined as the lowest concentration that there were no visible colonies. Experiments were performed in triplicate with different starting cultures. An example of MIC data is pictured in Figure 23.

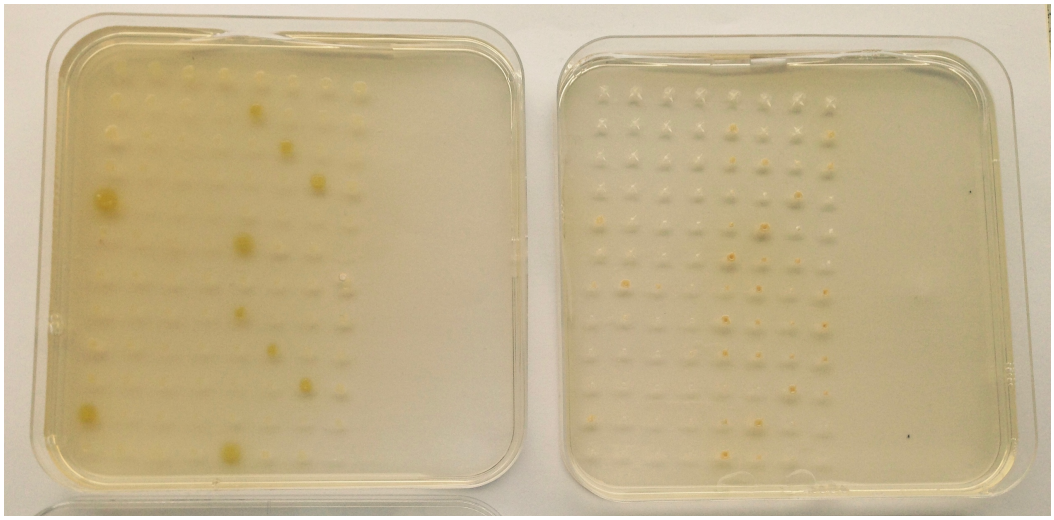


Figure 23: Agar dilution assay plates. The left plate is a pH5 BSM control agar plate with no preservative and the right plate is a pH5 BSM agar + 0.1% Benzoic Acid.

4.3.2 Preservative Susceptibility Testing of Four Bcc Strains to Nature identical and Synthetic Preservative Systems

From the larger collection of 40 *Burkholderia* industrial contaminant strains used previously, four Bcc strains were then selected for MIC determination by broth assay and subsequent microevolution (see section 4.3.4).

Preservative stock was added to BSM broth to a total volume of 4.5 ml in duplicate 28 ml plastic universal tubes. Stock solutions of preservative were added to BSM broth to achieve final concentrations ranging from: phenoxyethanol 0 – 0.1875% v/v, benzisolthiazolinone 0 – 0.0105% v/v, benzyl alcohol 0 – 0.328% v/v, benzoic acid 0 – 0.5% w/v, and salicylic acid 0 – 0.5% w/v.

Strains were cultured for 18 hours in 3 ml of BSM at 30°C in 28 ml sterile universals and shaken at 150 rpm. Approximately 1 x 10^5 CFU/ml of the test strains was added to the test and control (no preservative) universal tubes in duplicate and were incubated shaking (200 rpm) for 24 hours at 30 °C. The MIC was designated as the concentration of preservative at which there was an optical density matching the average turbidity reading of control universal tubes at 630 nm. Experiments were repeated three times with different starting cultures to obtain biological replicates.

4.3.3 Preservative Induced Adaptive Resistance

4.3.3.1 Strain and Preservative Selection

Within the collection the *B. cenocepacia* (N=9) species is the most common species in the industrial contaminant panel. Therefore, three *B. cenocepacia* strains with low and mid preservative MIC profiles were chosen. Due to its interesting MIC profile *B. lata* strain 1299 was also chosen.

Due to variability with pH 5 MIC's, bacterial microevolutions to the organic acids could not be performed.

Three preservatives were used as selective agents, two nature identical preservative agents (benzyl alcohol and phenoxyethanol) and the synthetic preservative benzisolthiazolinone. Benzisolthiazolinone was used as a control to evaluate the

success of different methodologies as this preservative has already been seen to cause adaptive resistance in *Burkholderia lata* strain 383 (Rushton *et al*., 2013).

4.3.3.2 High Concentration and Fast Timeframe (H/F) Adaptive Evolution Conditions

Broth evolution method

Plates were set up with duplicate two-fold dilutions of each preservative at 100, 70 and 30% strength. The initial stock solution concentration of preservatives was selected based on the results of the MIC assay and produced a set of doubling dilutions which encompassed the MIC value observed. All plates were incubated static at 30 °C. The initial inoculum was an overnight culture diluted to approximately $1x10^6$ CFU/ml. Bacteria from the duplicate wells with the highest concentration of preservative which supported growth consistent with the control after 24 hours was passaged forward to the next plate. All wells of subsequent passages were inoculated with a 1:1000 dilution of the bacteria passaged forward. This passaging was repeated for a period of 12 days or until three consecutive passages yielded no increase. Strains were stocked in 8% v/v DMSO at -80 °C after each passage. The T0 time point was also stocked as a comparator strain.

Agar evolution method

An agar evolution protocol was used for phenoxyethanol preservative only due to solubility issues. After autoclaving BSM agar was cooled to 50 °C and preservative stock was added to 20 ml of agar to produce a range of preservative plates equal to four-fold lower than the MIC and which increased 1.5 and 2-fold. A non-preserved control was also included. Strains were cultured for 18 hours in 3 ml of BSM broth as previously described. Plates were inoculated with 100 µl of 1 $x10^6$ CFU/ml and incubated for 48 hours at 30 °C. Bacteria from highest preservative concentration were inoculated into 3 ml of BSM broth and grown for 18 hours at 30 °C. The inoculum was then diluted 1/1000000 (approximately 1 $x10^3$ CFU/ml) and plated out again. Each broth passage was also stored in 8% v/v DMSO cryoprotectant for later analysis. The passaging was repeated until three consecutive passages yielded no increase.

4.3.3.3 Low Concentration and Slow Timeframe (L/S) Adaptive Evolution Conditions

Broth evolution method

Preservative stock was added to BSM broth to a total volume of 4.5 ml in 20 ml plastic universal tubes. Stock solutions of preservative were added to BSM broth to achieve final concentrations ranging from: phenoxyethanol 0.0139 – 0.3602% v/v, benzisolthiazolinone 0.00010 – 0.00312% v/v and benzyl alcohol 0.025 – 0.738% v/v.

Strains were cultured for 18 hours in 3 ml of BSM at 30°C in 28 ml sterile universals and shaken at 150 rpm. Cultures were diluted in sterile PBS to approximately 1 x 10^3 CFU and added to the test and control (no preservative) universal tubes in duplicate and incubated static for 24 hours at 30°C.

Stepwise evolution began with preservative concentration 6-fold lower than MIC and increased in 1.5-fold increments. Subsequent passages examined 1.5 and 3-fold lower and 1.5-fold higher than the previous preservative concentration.

A control (no preservative) was included with each passage. The average turbidity readings of the control universal tubes at 630 nm was used to define MIC point. MIC was defined as experimental turbidity readings with <10% the optical density of the control. Bacterial growth from both duplicates was combined and used as inoculum for the next set of concentrations. Bacteria was diluted in PBS buffer to approximately 1 x 10^3 CFU and added to test universals and incubated for 24-72 hours static at 30 °C. Microevolution continued until three consecutive passages yielded no increase.

4.3.4 Testing Stability of Preservative-Adapted *B. lata* and *B. cenocepacia* Strains

Stability of increased preservative resistance clones were confirmed by performing 10 successive passages onto BSM agar that did not contain preservative and repeated the MIC determination by broth assay (see section 4.3.2). Preservative

concentrations 1.5 and 3-fold above and below the MIC and terminal concentration of microevolution were used. Stocked strains were revived, and the experiments were repeated a total of three times with different starting cultures to obtain biological replicates.

4.4 Results

4.4.1 Preservative Susceptibility of a Panel of *Burkholderia* Strains to Nature identical and Synthetic Preservative Systems

Preservative susceptibility was calculated for a collection of seven clinical and 40 industrial *Burkholderia* isolates, against four synthetic and two nature identical preservative agents using an agar-dilution assay. There was a statistically significant difference for MIC values between preservative agents as determined by one-way ANOVA ($F(5,276) = 168.915$, $p = 0.0001$).

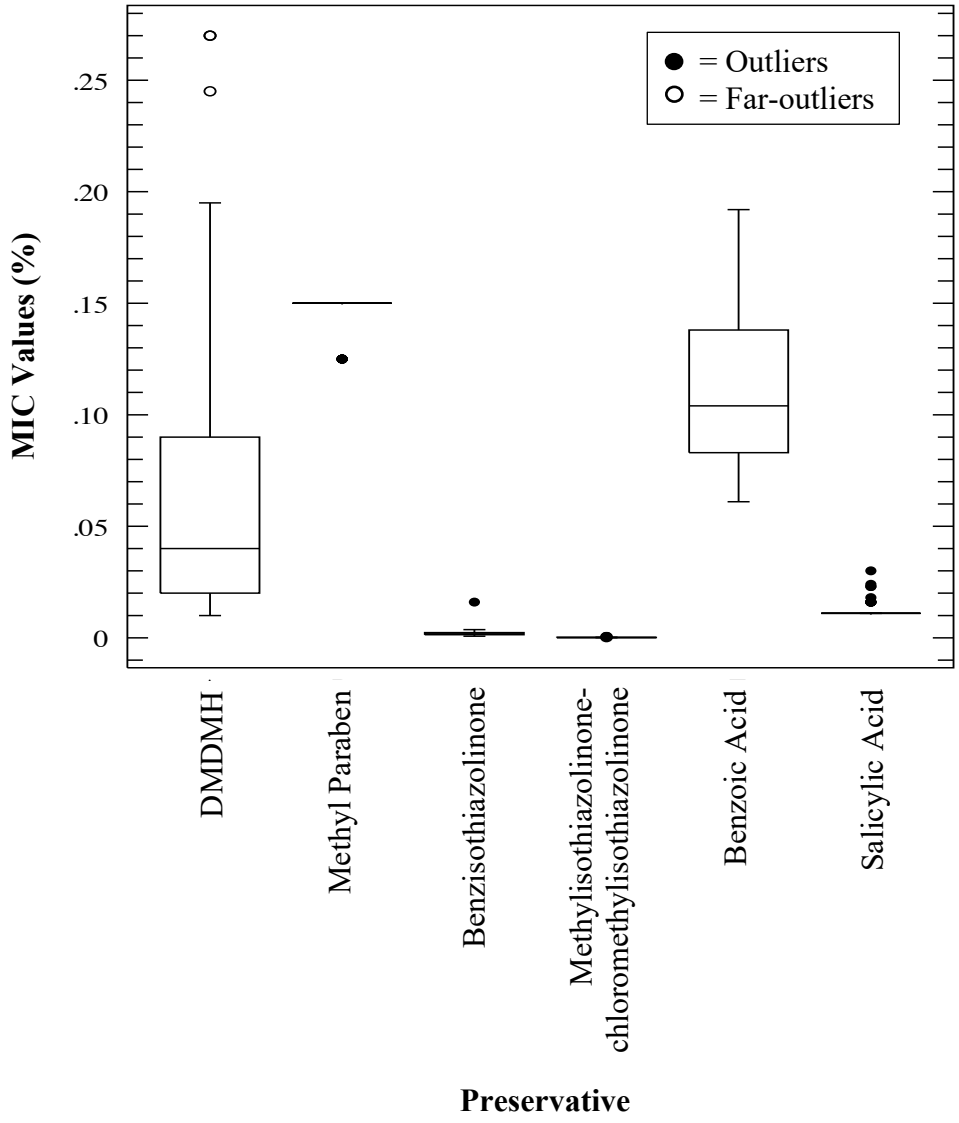


Figure 24: MIC values for four synthetic preservatives and two nature identical preservatives across the 47 tested *Burkholderia* species. The 4 synthetic preservatives were dimethylol dimethyl hydantoin, methyl paraben, benzisothiazolinone and methylisothiazolinone-chloromethylisothiazolinone. The two nature identical preservatives were benzoic acid and salicylic acid. MIC % refers to w/v for benzoic acid and salicylic acid and v/v for other agents. Whiskers indicate ranges and lines within boxes indicate the median.

Preservative susceptibility to the synthetic preservative DMDMH and the nature identical preservative benzoic acid varied significantly (Figure 24). The distribution of MIC values for DMDMH and benzoic acid were wide, while MIC values for the other agents clustered around the median. Standard deviation values for DMDMH (0.07) and benzoic acid (0.04), far exceeded that of the other preservatives tested (Table 14). The majority of *Burkholderia* strains tested shared a MIC value for methyl paraben (N=40/47, 0.150% v/v), and salicylic acid (N=36/47, 0.011% w/v).

MIC values for the isothiazolinone class of preservatives cluster together (Figure 24).

Table 14: Highest and Lowest MIC values (%), Standard Deviation and the Highest MIC as a Percentage of Regulated Amount of synthetic and nature identical preservatives for 47 *Burkholderia* species.

		Highest MIC (%)	Lowest MIC (%)	Standard Deviation (SD)	Highest MIC as Percentage of Regulated Amount (%)
Synthetic Preservatives	DMDMH	0.27	0.01	0.07	90
	MP	0.15	0.125	0.01	37.5
	BIT	0.016	0.0007	0.002	8
	M-CMIT	0.0006	0.00005	0.0001	40
Nature Identical Preservatives	BA	0.192	0.061	0.04	38.4
	SA	0.03	0.0113	0.004	6

Footnotes:
MIC % refers to w/v for benzoic acid and salicylic acid and v/v for other agents.
Abbreviations:
DMDMH, Dimethylol Dimethyl Hydantoin
MP, Methyl paraben
BIT, Benzisothiazolinone
M-CMIT, Methylisothiazolinone-chloromethylisothiazolinone
BA, Benzoic acid
SA, Salicylic acid

The highest observed MIC value and its percentage proportion of its regulated in-use amount is given in Table 14. The synthetic preservative DMDMH required the highest inhibitory concentration (0.27% v/v) and this account for 90% percent of the regulated in-use concentration. Compared to the highest observed MIC value of DMDMH, the highest observed MIC value for the nature identical preservatives benzoic acid (0.192% w/v) and salicylic acid (0.03% w/v) were 1.4-fold and 9-fold lower respectively. The highest observed MIC value of the synthetic preservative methyl paraben (0.150% v/v) was 5-fold greater than the highest MIC value for the nature identical preservative salicylic acid (0.03% w/v) and only 0.78-fold lower than the highest MIC for benzoic acid (0.192% w/v).

The biggest difference between MIC values of synthetic and nature identical preservatives is observed between the isothiazolinone class (Figure 24). The highest MIC value of the nature identical preservative benzoic acid (0.192% w/v) was 12-

fold greater than the highest MIC value for benzisothiazolinone concentration (0.016% v/v) and 32-fold greater than the highest MIC for methylisothiazolinone-chloromethylisothiazolinone (0.006% v/v) (Table 14). The highest MIC value of nature identical preservative salicylic acid (0.03% w/v) was 1.8-fold greater than the highest MIC value for benzisothiazolinone (0.016% v/v) and 5-fold greater than the highest MIC value for methylisothiazolinone-chloromethylisothiazolinone (0.006% v/v) (Table 14).

Preservative susceptibility to nature identical and synthetic preservatives between and within *Burkholderia* groups were highly variable (Figure 25). Statistical analysis of species-dependent differences in preservative susceptibility as nine out of eleven species groups contained five or less representative strains. *Burkholderia* groups and strains exhibited lower susceptibility for certain preservatives but not for others (Table 15). The highest MIC for the preservative salicylic acid was observed by the species group *B. multivirons* (0.0154% w/v) however this group also had the lowest MIC for the preservatives M-CMIT (0.00007% v/v) and benzoic acid (0.068% w/v). For preservatives related to the same class, there was also no consensus between MIC values. For example, *B. multivirons* strain 1559 MIC values for the organic acid preservative group varied significantly, with the lowest observed MIC value for benzoic acid (0.061% w/v) and the second highest for salicylic acid (0.024% w/v).

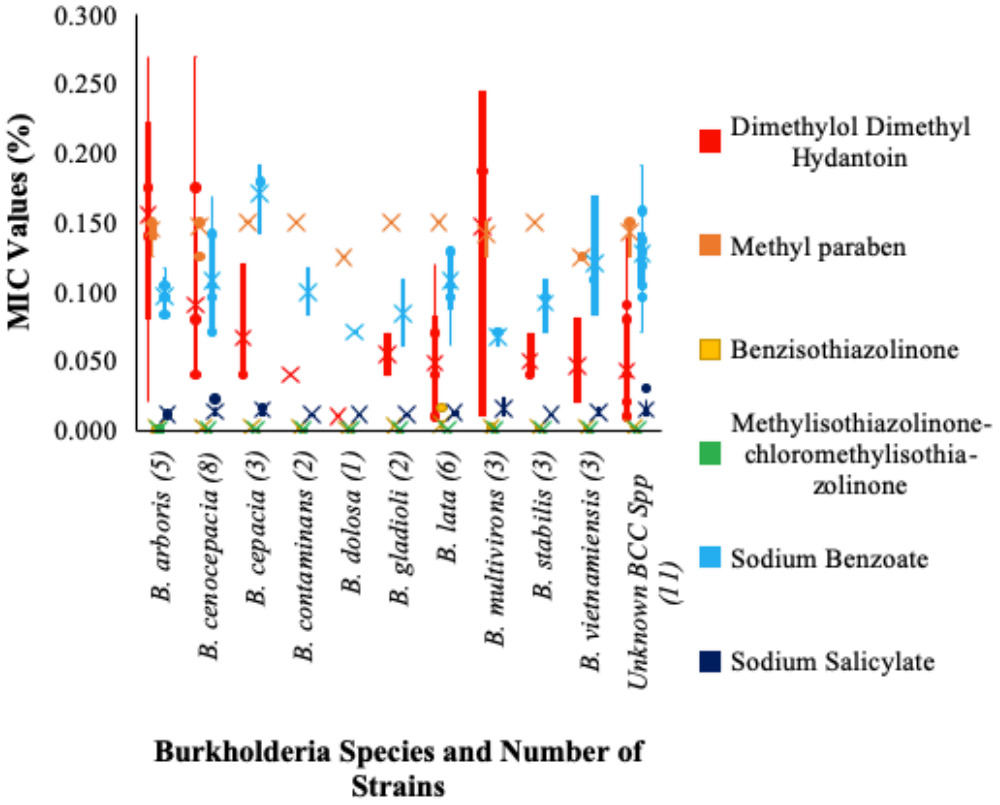


Figure 25: Distribution of MIC values for four synthetic preservatives and two nature identical preservatives across the 11 *Burkholderia* species/groups. The 4 synthetic preservatives were dimethylol dimethyl hydantoin, methyl paraben, benzisothiazolinone and methylisothiazolinone-chloromethylisothiazolinone. The two nature identical preservatives were benzoic acid and salicylic acid. MIC % refers to w/v for benzoic acid and salicylic acid and v/v for other agents. Whiskers indicate ranges, crosses indicate means and dots indicate outliers.

MIC values for dimethylol dimethyl hydantoin, methyl paraben, benzisothiazolinone, methylisothiazolinone-chloromethylisothiazolinone, benzoic acid and salicylic acid did not exceed the maximum permitted amount (Table 3). However, MIC values for three strains were very close to the permitted amount of the preservative dimethylol dimethyl hydantoin (0.3% v/v): *B. arboris* strain 1310, (0.27% v/v), *B. cenocepacia* strain 1558 (0.27% v/v) and *B. multivirons* strain 1559 (0.25% v/v).

B. lata strain 1299 exhibited much lower susceptibility to the preservative benzisothiazolinone. The highest average MIC concentration for the preservative was 0.0160% v/v, 4.5 times greater than the next highest concentration (0.0035% v/v).

Table 15: Mean and range Minimum Inhibitory Concentrations (%) values of synthetic and nature identical preservatives for 47 *Burkholderia* strains.

	Minimum Inhibitory Concentrations (%)											
	Synthetic Preservatives								Nature identical Preservatives			
	Formaldehyde Donor		Paraben		Isothiazolinone				Organic Acid			
	DMDMH		Methyl paraben		BIT		M-CMIT		Benzoic acid		Salicylic acid	
Species Group and Number of Strains	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
B. arboris (5)	0.156	0.02 - 0.27	0.15	0.125 - 0.150	0.00182	0.00085 - 0.0035	0.00011	0.00005 - 0.00015	0.097	0.083- 0.117	0.0113	0.0113 - 0.0113
B. cenocepacia (8)	0.088	0.04 - 0.27	0.15	0.125 - 0.150	0.00197	0.00135 - 0.0035	0.00022	0.00005 - 0.0006	0.110	0.071- 0.169	0.0131	0.0113 - 0.0225
B. cepacia (3)	0.07	0.04 - 0.12	0.15	0.150 - 0.150	0.00233	0.00175 - 0.0035	0.00011	0.00005 - 0.00015	0.171	0.142 - 0.192	0.0146	0.0113 - 0.0163
B. contaminans (2)	0.04	0.04 - 0.04	0.15	0.150 - 0.150	0.00155	0.00135 - 0.00175	0.00013	0.0001 - 0.00015	0.100	0.083 - 0.117	0.0113	0.0113 -0.0113
B. dolosa (1)	0.01		0.13		0.00085		0.00013		0.071		0.0113	
B. gladioli (2)	0.06	0.04 - 0.07	0.15	0.150 - 0.150	0.00325	0.003 - 0.0035	0.00010	0.0001 - 0.0001	0.085	0.061- 0.108	0.0113	0.0113 -0.0113
B. lata (6)	0.04	0.01 - 0.12	0.15	0.150 - 0.150	0.00495	0.00125 - 0.016	0.00017	0.000075 - 0.00045	0.104	0.061 - 0.129	0.0113	0.0113 - 0.0175
B. multivirons (3)	0.148	0.01 - 0.25	0.14	0.125 - 0.150	0.00163	0.00065 - 0.0025	0.00007	0.00005 - 0.0001	0.068	0.061- 0.071	0.0154	0.0113 - 0.0238
B. stabilis (3)	0.05	0.04 - 0.07	0.15	0.150 - 0.150	0.00175	0.00125 - 0.00225	0.00010	0.0001 - 0.0001	0.092	0.071 - 0.108	0.0113	0.0113 - 0.0113
B. vietnamiensis (3)	0.05	0.02 - 0.08	0.13	0.125 - 0.125	0.00148	0.00125 - 0.00185	0.00010	0.00005 - 0.00015	0.120	0.083 - 0.169	0.0129	0.0113 - 0.0163
Unknown Bcc *Species* (11)	0.04	0.01 - 0.14	0.14	0.125 - 0.150	0.00195	0.00125 - 0.0035	0.00011	0.00005 - 0.0003	0.127	0.071 - 0.192	0.0149	0.0113- 0.030

Footnotes:
MIC % refers to w/v for benzoic acid and salicylic acid and v/v for other agents
Abbreviations:
DMDMH, Dimethylol dimethyl hydantoin
BIT, Benzisothiazolinone
M-CMIT, Methylisothiazolinone-chloromethylisothiazolinone

There were 40 industrial strains and seven clinical strains in the panel of 47 *Burkholderia* strains tested against preservatives. The 40 industrial strains represent nine of the Bcc species groups, a closely related *Burkholderia* strain *B. gladioli* and uncharacterised Bcc strains. The seven clinical strains represent uncharacterised Bcc strains the three of the Bcc species groups.

Burkholderia environmental-industrial isolates had a greater mean MIC concentration to specific preservatives than *Burkholderia* isolates from clinical sources (Figure 26). Across the six preservatives tested, there was a significant difference in preservative susceptibility to methyl paraben between *Burkholderia* environmental-industrial (M=0.148, SD=0.008) and *Burkholderia* clinical isolates (M=0.129, SD=0.009); t (45)= 5.8720, p = 0.001 (Table 16).

Table 16: Preservative MIC values (%) for *Burkholderia* isolated from industrial sources (N=40) and clinical sources (N=7)

		Group Statistics		T-test Statistics			
Preservative	**Isolate Source**	**Mean MIC Value (%)**	**Standard Deviation**	**P Value**	**t**	**df**	**Standard Error of Difference**
DMDMH	Industrial	0.079	0.070	0.115	1.6081	45	0.028
	Clinical	0.034	0.062				
MP	Industrial	0.148	0.008	0.001	5.8720	45	0.003
	Clinical	0.129	0.009				
BIT	Industrial	0.0024	0.0023	0.387	0.8736	45	0.001
	Clinical	0.0016	0.0005				
M-CMIT	Industrial	0.00014	0.00013	0.124	1.5658	45	<0.000
	Clinical	0.00007	0.00003				
BA	Industrial	0.113	0.036	0.179	1.3637	45	0.014
	Clinical	0.093	0.030				
SA	Industrial	0.0134	0.0044	0.400	0.8496	45	0.002
	Clinical	0.0120	0.0019				

Footnotes:
MIC % refers to w/v for benzoic acid and salicylic acid and v/v for other agents
Abbreviations:
DMDMH, Dimethylol dimethyl hydantoin
MP, Methyl paraben
BIT, Benzisothiazolinone
M-CMIT, Methylisothiazolinone-chloromethylisothiazolinone
BA, Benzoic acid
SA, Salicylic acid

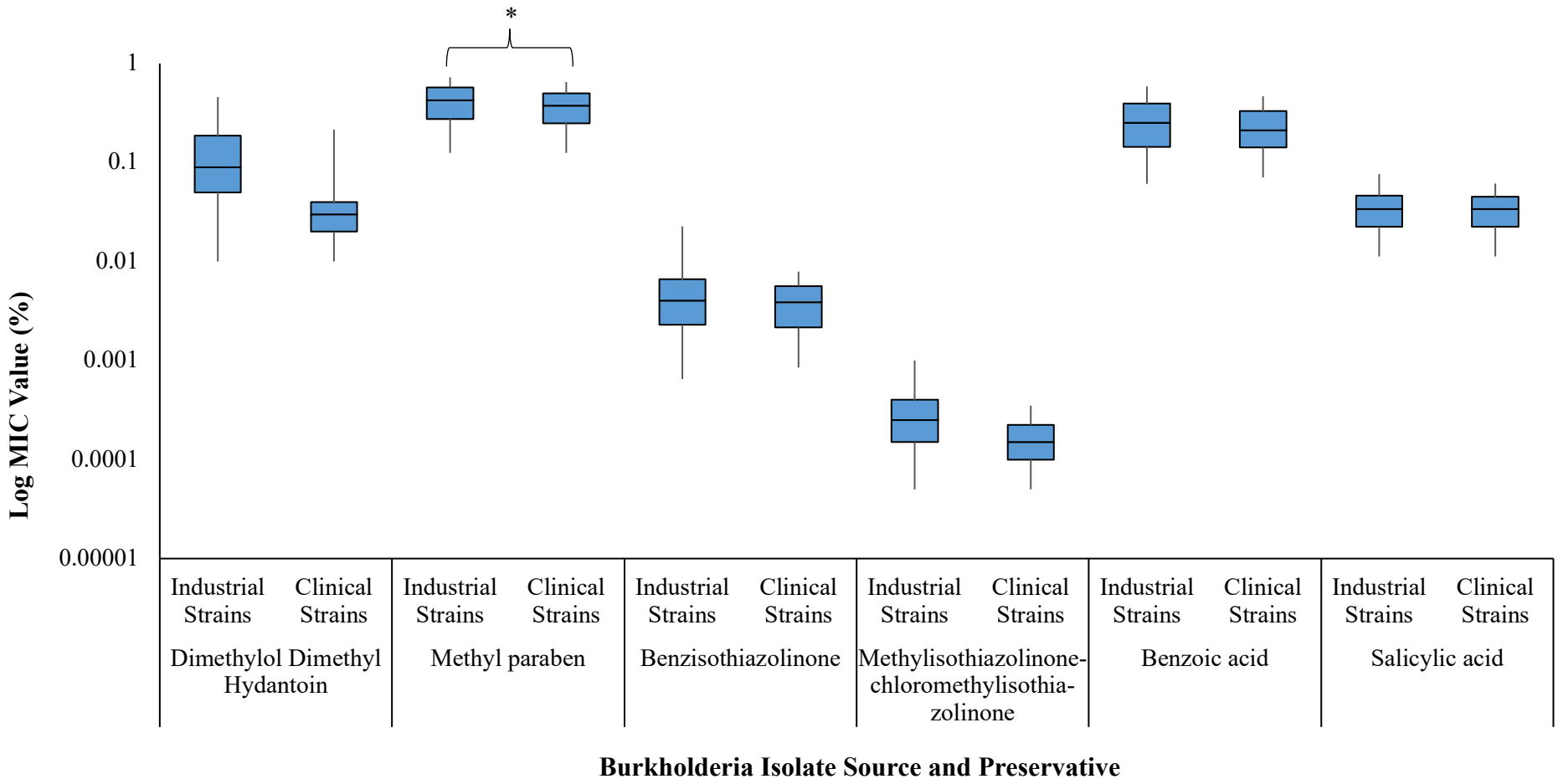


Figure 26: LOG MIC values (%) for industrial (N=40) and clinical (N=7) isolates of *Burkholderia* species challenged with six preservatives. MIC % refers to w/v for benzoic acid and salicylic acid and v/v for other agents. Whiskers indicate ranges and box lines indicate the median. Statistically significant results are indicated with a (*).

4.3.2 Preservative Susceptibility Testing of Four Bcc Strains to Nature identical and Synthetic Preservative Systems

In the panel of environmental-industrial isolates the relative species abundance was highest for *B. cenocepacia* therefore three *B. cenocepacia* strains with low-average preservative susceptibility were chosen for further examination. Due to its ability to tolerate high concentrations of the preservative BIT *B. lata* strain 1299 was also chosen for further examination.

MIC values for benzyl alcohol, BIT, phenoxyethanol, salicylic acid and benzoic acid were then repeated using MIC broth assay methods with four Bcc strains (Figure 27). MIC values of the four Bcc strains and the preservatives benzyl alcohol and phenoxyethanol were similar, ranging from 0.1244 - 0.2188% v/v and 0.0702-0.1064% v/v respectively. MIC's for *B. cenocepacia* strains and the preservative BIT were similar and ranged from 0.00074 – 0.00092% v/v. *B. lata* strain 1299 demonstrated a much lower preservative susceptibility, with a MIC an order of magnitude higher (0.00648% v/v).

MIC's for *B. lata* strain 1299 in the organic acid preservatives benzoic acid and salicylic acid could not be produced as this strain was not viable at pH 5, which is required for preservative activity. Average MIC values for *B. cenocepacia* strains ranged from 0.256% - 0.273% w/v for benzoic acid and 0.0303% - 0.0335% w/v for salicylic acid (Figure 27). There was a wide variability within each strain MIC values, as seen in figure 27. This could be caused by variation's in pH and dilution issues. Due to this variability these compounds were not used for adaptive resistance experiments.

MIC broth microdilution assay using one synthetic (BIT) and four nature identical preservatives (benzyl alcohol, phenoxyethanol, salicylic acid and benzoic acid) found that the synthetic preservative was most effective against *Burkholderia* strains at the lowest concentration levels (Table 17). BIT was effective at concentrations 0.37 - 3.24% v/v of the regulated amount. Concentrations for nature identical preservatives varied by preservative type and were not similar by preservative class. The percentage of regulated amount was the highest for the nature identical preservative benzoic acid (51.2 - 54.6% w/v) and benzyl alcohol (12.4 - 21.8% v/v).

Due to the high concentration required for the efficacy these preservatives would be very sensitive to dilution effects and could potentially encourage adaptive resistance responses. The percentage of regulated amount was the lowest for the nature identical preservatives phenoxyethanol (7 - 10.6% v/v) and salicylic acid (6 - 6.7% w/v). Due to the lower concentrations required for efficacy these nature identical preservatives would be less sensitive to dilution effects and less likely to encourage adaptive resistance responses.

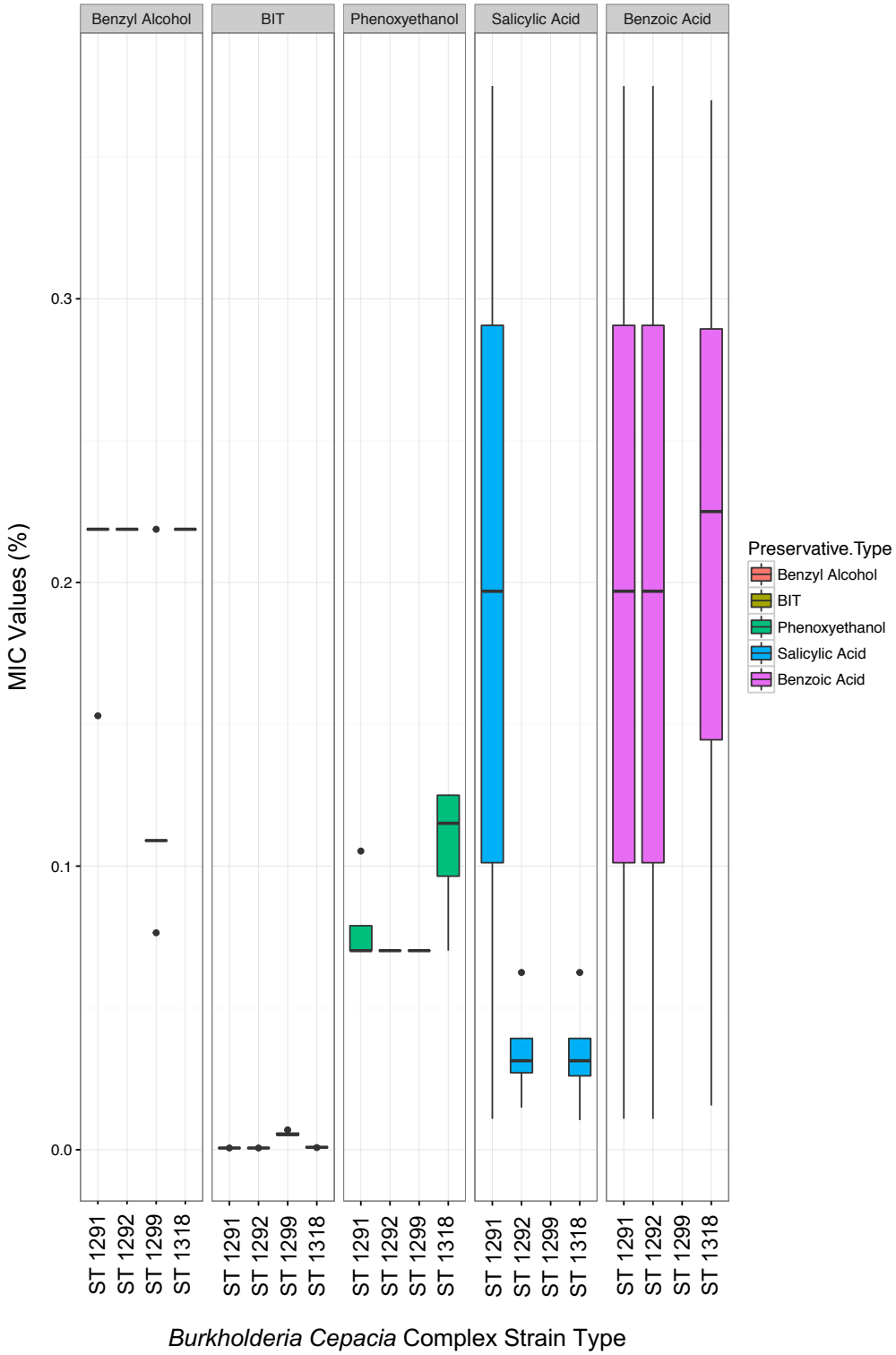


Figure 27: MIC values (%) for benzyl alcohol, BIT, phenoxyethanol, salicylic acid and benzoic acid and *B. cenocepacia* strains 1291, 1292, and 1318, and *B. lata* strain 1299. MIC % refers to w/v for benzoic acid and salicylic acid and v/v for other agents.

4.4.3 Preservative Induced Adaptive Resistance

In both (H/F) and (L/S) adaptive resistance experiments MIC values for benzisothiazolinone, benzyl alcohol and phenoxyethanol did not exceed the maximum permitted amount (Table 3).

Adaptive resistance to nature identical preservatives varied. Sub-inhibitory benzyl alcohol did not increase MIC values during H/F and L/S experiments with the exception of one L/S experiment with *B. lata* strain 1299 which demonstrated decreased preservative susceptibility of 1.5-fold (Table 17). Whereas for the nature identical preservative phenoxyethanol, in both H/F and L/S experiments all Bcc strains were able to reduce preservative susceptibility from concentrations previously observed in MIC's (Table 17).

For the synthetic preservative BIT most Bcc strains were able to decrease preservative susceptibility from concentrations previously observed in MIC's with the exception of the already BIT tolerant with *B. lata* strain 1299 (Table 17). During H/F experiments adaptive resistance to BIT was acquired quickly by *B. cenocepacia* strains, surpassing previously observed MIC concentrations after 2-3 passages (Figure 28).

Table 17: Fold change of preservative Minimum Inhibitory Concentrations values (%) for *Burkholderia cepacia* complex strains after adaptive resistance experiments. Adaptive resistance experiments were performed using two experimental methods "Low concentration and Slow timeframe" and "High concentration and Fast timeframe".

Preservative	Experiment Method	*B. cenocepacia*			*B. lata*
		1291	1292	1318	1299
Benzyl Alcohol	H/F	0	0	0	0
	L/S	0	0	0	1.5
Phenoxyethanol	H/F	2*	2*	1.5*	2*
	L/S	3*	3*	1.5*	3*
BIT	H/F	1.5*	1.5*	1.5*	0
	L/S	3*	3*	3*	1.5

Footnotes:
* indicates stable fold changes
Abbreviations:
H/F, High and Fast
L/S, Low and Slow

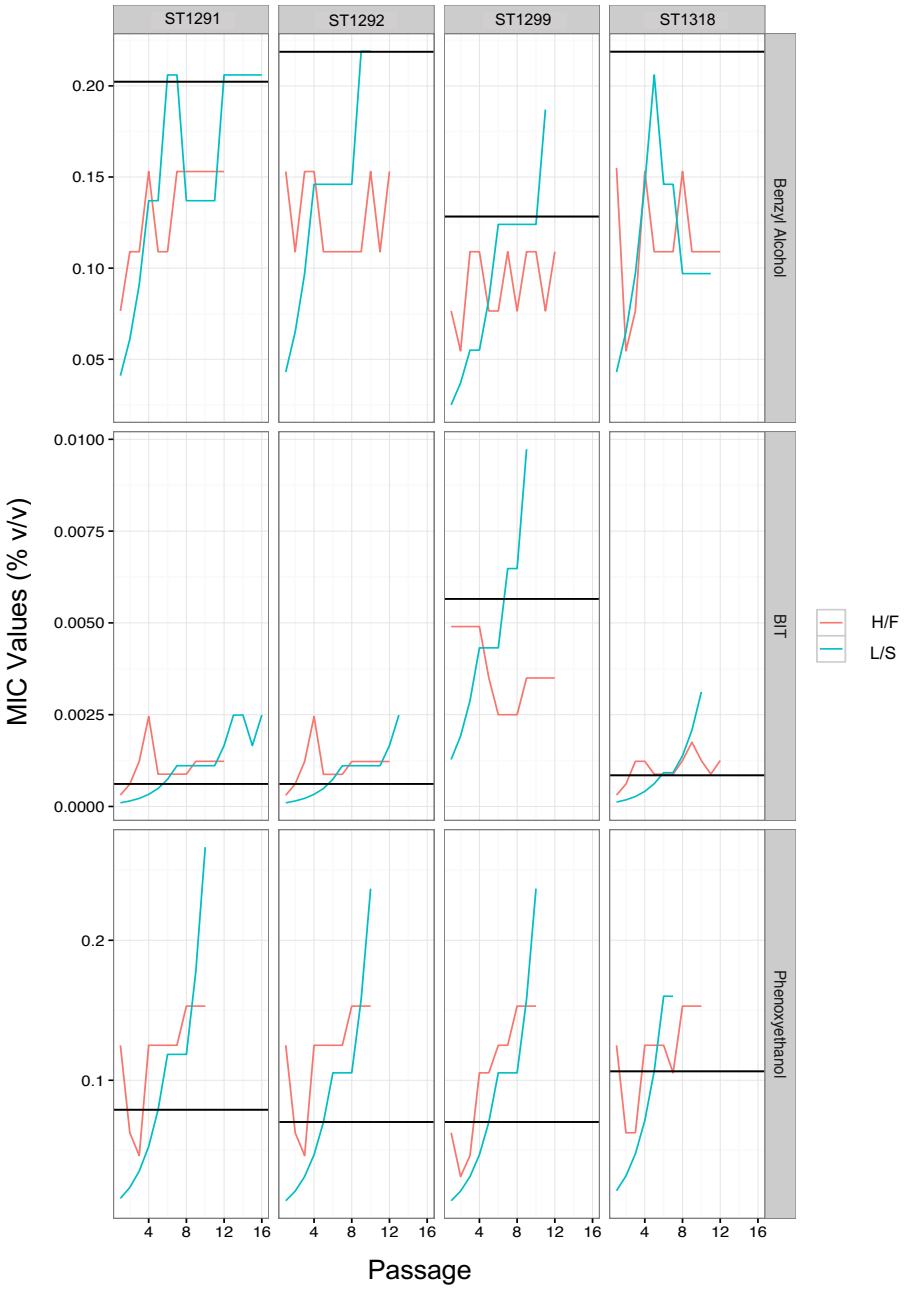


Figure 28: Benzyl alcohol, BIT, phenoxyethanol MIC data by passage for "Low and Slow" and "High and Fast" evolution strategies using *B. cenocepacia* strains 1291, 1292, and 1299, and *B. lata* strain 1299. Previously observed MIC's for each preservative and strain are represented by a solid black line.

4.4.3.1 High Concentration and Fast Timeframe (H/F) and Low Concentration and Slow Timeframe (L/S) Adaptive Evolution Conditions

The choice of preservative concentration and exposure method did affect preservative susceptibility. Preservative susceptibility above MIC levels was seen more widely with L/S rather than H/F adaptive resistance experiments. In benzyl alcohol experiments, all strains failed to reach previously seen MIC values for H/F adaptive resistance experiments however in L/S adaptive resistance experiments three out of four strains met or surpassed MIC concentrations (Figure 28).

For H/F resistance experiments increases in MIC fluctuated passage to passage (Figures 28). For most adaptive resistance (L/S) experiments preservative resistance increased linearly passage on passage (Figure 28). The exception to this was benzyl alcohol preservative and *B. cenocepacia* strain 1291 and *B. cenocepacia* strain 1318 (Figure 28). *B. cenocepacia* strain 1291 fluctuated between 1.5-fold decrease in MIC and the MIC. *B. cenocepacia* strain 1318 displayed increased preservative susceptibility after the 6^{th} passage but successive passages resulted in a decrease of 2-fold below MIC values.

4.3.4 Testing Stability of Preservative-Adapted *B. lata* and *B. cenocepacia* Strains

Repeat MIC's were performed after 10 successive passages (P10) in the absence of preservative to see if MIC levels returned to original values or if decreased preservative susceptibility was retained. Stable changes in MIC values are given in Table 17.

Both H/F and L/S adaptive resistance experiments with benzyl alcohol preservative did not reach MIC levels with the exception of *B. lata* strain 1299 which displayed mild increases in MIC (1.5-fold) however P10 MIC was the same as the original MIC, loosing phenotypic resistance (Table 17).

For H/F and L/S adaptive resistance experiments with phenoxyethanol preservative all Bcc strains exceeded original MIC levels demonstrated a stable increase in preservative resistance (Table 17).

For BIT preservative and both H/F and L/S adaptive resistance experiments, *B. cenocepacia* strains exceeded MIC levels during experiments and the P10 MIC also exceed the original MIC (Table 17). For H/F adaptive resistance experiments, *B. lata* strain 1299 failed to reach MIC levels during experiments and the P10 MIC (0.0035% v/v) was two-fold lower than the original MIC (0.00648% v/v). For L/S adaptive resistance experiments, *B. lata* strain 1299 exceeded MIC levels 1.5-fold during experiments however the P10 MIC (0.0050% v/v) was similar to the original MIC (0.00648% v/v).

MIC values were lower for synthetic preservatives than nature identical preservatives. Despite increases in synthetic preservative resistance the percentage of regulated amount did not increase significantly and remained extremely low (Table 18). Before successive passage, benzyl alcohol preservative required the highest concentration out of both nature identical preservatives to inhibit *Burkholderia* growth. However, after adaptive resistance experiments the percentage of regulated amount increased in both benzyl alcohol (15.3- 25%) and phenoxyethanol (15.3-23.3%) to similar levels.

Table 18: MIC (%) range values of nature identical preservatives and synthetic preservatives for *B. cenocepacia* strains 1291, 1292, and 1318, and *B. lata* strain 1299 and their relative percentage of the regulated amount before and after successive passage in preservatives.

		Regulated Amount (%)	MIC (%)	Percentage of Regulated Amount (%)	MIC After Successive Subculture (%)	Percentage of Regulated Amount (%)
Nature identical Preservatives	Benzyl alcohol	1.0	0.124 - 0.219	12.4 - 21.9	0.153 - 0.25	15.3- 25
	Phenoxyethanol	1.0	0.0702 - 0.1064	7 - 10.6	0.153 - 0.233	15.3 - 23.3
	Benzoic acid	0.5	0.256 - 0.273	51.2 - 54.6	N/A	N/A
	Salicylic acid	0.5	0.0303 - 0.0335	6 - 6.7	N/A	N/A
Synthetic Preservatives	BIT	0.2	0.00074 - 0.00648	0.37 - 3.24	0.00145 - 0.0049	0.7 – 2.45

4.4 Discussion

4.4.1 Preservative Susceptibility Testing

4.4.1.1 Comparing Nature identical Preservatives and Synthetic Preservatives

Preservative susceptibility testing was conducted using of four different nature identical preservatives, benzoic acid, salicylic acid, phenoxyethanol and benzyl alcohol. In this study, nature identical preservatives were effective against all tested *Burkholderia* strains at levels permitted by the European Union (EU) Council Directive 76/768/EEC for cosmetics and personal care products (Table 14 and 18).

Preservative susceptibility testing performed with a collection of 47 *Burkholderia* strains found that organic acid nature identical preservatives (benzoic acid, salicylic acid) demonstrated greater efficacy at lower concentrations against *Burkholderia* strains than the synthetic preservative DMDMH. The maximum observed MIC of DMDMH was 1.4 times higher than benzoic acid and 9 times higher than salicylic acid (Table 14). Furthermore, the greatest MIC for the synthetic preservative methyl paraben was 5 times higher than that of salicylic acid and similar to benzoic acid (Table 14). Therefore, in concordance with recent research (Glavač and Lunder, 2018) nature identical preservatives can demonstrate efficacy against gram-negative contaminant species such as *Burkholderia* in concentrations equal or lower to specific synthetic preservatives.

Comparative evaluations of the levels of synthetic or nature identical preservative in relation to their permitted in use concentrations were performed (Table 14 and 18). Agents used at lower levels in relation to the permitted values would lower the potential for adaptive resistance at higher levels. The nature identical preservatives salicylic acid (6.7%), benzyl alcohol (21.9%) and phenoxyethanol (10.9%) were effective against *Burkholderia* strains at the lowest levels in relation to its permitted in-use concentration than synthetic preservatives DMDMH (90%), methyl paraben (37.5%), M-CMIT (40%). Therefore, some nature identical preservatives demonstrate a lower potential for the development of preservative adaptive resistance than synthetic preservatives.

B. cenocepacia resistance to the nature identical preservative benzoic acid was high and required concentrations of half the regulated amount to effectively inhibit Bcc growth (Table 18). Although the mechanisms behind decreased susceptibility are unclear there is evidence from that bacterial species can synthesise and metabolise benzoic acids (Beloborodova, 2016). Furthermore, various environmental-industrial *Pseudomonas* species have been seen to degrade benzoic acid preservatives (Cao and Loh, 2008; Nichols and Harwood, 1995; Thayer and Wheelis, 1982). Therefore increased intrinsic resistance to benzoic acid preservatives in Bcc strains could be caused by the extensive metabolic capabilities of these organisms.

4.4.1.2 Comparing Environmental-Industrial Isolates and Clinical Isolates

This study utilised genomically diverse environmental-industrial and clinical *Burkholderia* strains including 8 Bcc species groups. Environmental-industrial *Burkholderia* strains were more tolerant to the common synthetic preservative methyl paraben than isolates from clinical sources (Table 16). Furthermore, three environmental-industrial isolates of *Burkholderia* exhibited preservative resistance close to regulated in use levels of the synthetic preservative DMDMH (Table 14). Although the cause of this intrinsic preservative resistance is unknown it has been suggested that industrial wild type strains have diverse metabolic activity and stress responses and may be pre-adapted to biocidal compounds which decreases antimicrobial susceptibility (Orth *et al*., 2006). This has been experimentally demonstrated, whereby *B. cepacia* contamination isolates exhibited a higher preservative resistance (up to 10-fold) than other *Burkholderia* strains (Orús *et al*., 2015; Rushton *et al*., 2013; Zani *et al*., 1997). Current preservative susceptibility is traditionally performed with ATCC type strains however, there is an awareness that these may underestimate the preservative susceptibility of environmental-industrially isolated micro-organisms. The observed increase in preservative resistance of methyl paraben and DMDMH preservatives for environmental-industrial *Burkholderia* strains compared to clinical demonstrates the importance of using contaminant test strains, as these are potentially more tolerant to preservatives than bacterial strains which have not had any exposure to personal care products or active preservatives.

Preservative susceptibility varied greatly across *Burkholderia* species and strains (Figure 25). Pan preservative resistance to all or specific classes of preservatives was not seen indicating that mechanisms of preservative resistance are agent and/or species specific. The phenomenon of biological variance to biocide resistance has been well studied, with considerable differences dependent on strain, antimicrobial agent and test methodology (Heinsel, 1998). The wide variability for preservative susceptibility across Bcc strains has also been demonstrated previously (Rushton *et al*., 2013). Preservative susceptibility varied within the same preservative class with a similar mode of action. For example, chloromethylisothiazolinone and benzisothiazolinone of the isothiazolinone class of preservatives are believed to have a similar mode of action. However, the unknown Bcc strain 1300 had the third lowest MIC for BIT (0.0013% v/v) and but the third highest for M-CMIT (0.00030% v/v). A lack of species dependent resistance to preservatives, and consensus between preservatives of the same class has also been demonstrated in clinical and industrial *Burkholderia* species (Rushton *et al*., 2013). One hypothesis for this phenomenon is that although they cause enzymatic disruption once inside the cell differences in uptake/cellular permeability change MIC values.

Previous studies have demonstrated that along with being the predominant Bcc species group isolated from the industrial environments, *B. cenocepacia* strains also exhibit high levels of resistance to preservatives and antibiotics (Rushton *et al*., 2013; Rose *et al*., 2009). In concordance with those findings, this study found the *B. cenocepacia* industrial contaminant strain 1558 had one of the largest MIC values for DMDMH, BIT (2nd largest), sodium benzoate (4th largest) and sodium salicylate (3rd largest) (Table 15).

Preservative susceptibility testing in broth microdilution could not be performed for organic acid preservatives and *B. lata* strain 1299 due to growth inhibition at pH 5, which is required for the preservative activity (Figure 27). This was not observed with *B. cenocepacia* strains in pH 5 media. Variability in viability and growth of *B. lata* strains has been identified previously, confounding antibiotic and preservative susceptibility testing (Cowley, 2016). *B. cenocepacia* is the most prevalent infection causing species in CF patients in European countries (Scoffone *et al*., 2017). The airway environment of CF persons has been found to be acidic (Tate *et al*., 2002;

Welsh and Smith, 1993), therefore Bcc species ability to tolerate acid stress essential for host colonisation and pathogenicity. Acid tolerance has also been identified in environmental-industrial Bcc isolates, with strains recovered as contaminants from products with a pH of 3 (Borovian, 1983). It is likely that as *B. cenocepacia* is a highly acid resistant Bcc species and is able to adapt quickly to acid environments.

4.4.2 Adaptive Resistance Testing

Adaptive resistance is the phenotypic change in a micro-organism ability to resist the toxic effects of an antimicrobial through exposure to a concentration gradient of that antimicrobial compound (Rizi *et al*., 2018). The induction and stability of antimicrobial adaptive resistance is thought to affected by various factors including exposure method and/or duration, strain and agent (Fernández *et al*., 2011). In this study, two methodologies were designed: A high and fast (H/F) protocol using the highest concentrations of preservative in fast successive passages (24-48 hours) and the low and slow (L/S) protocol using sub-inhibitory concentrations in slow passages (24-72 hours). It was hypothesised that manipulation of timeframe and antimicrobial gradient could induce different mechanisms of preservative adaptive resistance which could affect stability. Whilst the stability of adaptive resistance could not be correlated to the methodologies, higher levels of preservative resistance was consistently seen with L/S experiments when compared to H/F experiments (Table 17). This indicates sub inhibitory gradients and moderate selective pressure enables a higher level of preservative resistance. The incorporation of antimicrobial gradients in stepwise adaptive resistance training has previously seen to be important for adaptive resistance in other studies, for example one study found *E.coli* was able to increase MIC 3000 fold for trimethoprim and 20,000 fold for ciprofloxacin when grown on various concentrations increasing in orders of magnitude however, when bacteria were inoculated on plates with only one intermediary concentration, none were able to adapt to concentrations previously seen (Baym *et al*., 2016). Although the cause of this is unclear, it is hypothesised that long exposure to sub-inhibitory concentration gradients enables the development of compensatory mechanisms to cope with the fitness cost of adaptive resistance (Motta and Aldana, 2016).

Differences in the methodologies can also been seen in passage to passage. In H/F adaptive resistance experiments MIC values fluctuated passage to passage whereas

for L/S adaptive experiments MIC's increased in a steady linear manor (Figure 28). Differences seen in the passage-passage preservative changes could demonstrate how adaption through H/F and L/S methodologies, and the exertion of different selection pressure is likely driving differing genomic mutational changes. Other studies have observed differing passage to passage patterns and correlated this to differing mutations and mechanisms of adaptive resistance. In antibiotic stepwise training of *E. coli*, "jumping" increments relate to mutations in antibiotic specific target whilst strains that gained resistance "smoothly" had SNPs predominantly associated with fitness such as transcription/translation, protein biosynthesis and cellular membrane (Toprak *et al*., 2012). Although the exact molecular mechanisms will be explored in Chapter 5, this supports the observation that L/S methodologies are associated with greater preservative resistance, largely due to the reduced/compensatory fitness mechanisms which can develop to compensate mutations.

Adaptive resistance to benzyl alcohol preservative could not be promoted in *B. cenocepacia* strains and the preservative effectively inhibited growth at MIC levels. Transient adaptive resistance to benzyl alcohol preservative was induced in *B. lata* strain 1299, however this was most likely caused by a low initial MIC as the increase was modest (1.5 fold) and did not exceed concentration tolerated by other Bcc strains (Table 17). Successive passage in the preservative benzyl alcohol with *B. cenocepacia* strain *1318* resulted in increased preservative susceptibility and decreased survival (Figure 28). Whereas stepwise training with the two more common personal care product preservative systems, phenoxyethanol and BIT and resulted MIC increases for all four Bcc strains. Although the cause of decreased preservative susceptibility to benzyl alcohol is unknown, various studies which have reported decrease in biocide susceptibility through stepwise training hypothesise that it is due to bacterial fitness costs, as the organisms must divert metabolic resources and increase mutation rates (Pagedar *et al*., 2012; Soumet *et al*., 2012; Alonso-Hernando *et al*., 2009; Langsrud *et al*., 2004; McBain *et al*., 2004). This has been demonstrated experimentally where overexpression of biocide resistance mechanisms resulted in decrease in growth and survival (Wales and Davies, 2015). Furthermore, research suggests that the timeframe required for adaption to biocides is dependent on the commonality of the agent. Resistance to the common biocidal

agent Triclosan occurred after a short exposure (5 minutes) and caused a significant increase in MIC values 39 to 69-fold whereas exposure to the relatively uncommon bactericidal agent hydrogen peroxide caused decreases in resistance after short exposure and a modest (2 fold) increase in resistance after a 24hr period (Wesgate *et al*., 2016). Therefore, it is likely that as benzyl alcohol is an uncommon personal care preservative agent formation of preservative induced adaptive resistance mechanisms in the timeframes examined were not observed. This demonstrates that exploring novel alternatives for preservative systems could partially alleviate the growing issue of preservative resistance in personal care contaminant species.

This is the first study in which adaptive resistance to phenoxyethanol preservatives using Bcc strains have been seen. Phenoxyethanol adaptive resistance has been developed in various other species including *P. aeruginosa*, *E. coli* and *S. aureus* using progressive subculture in sub inhibitory conditions (Zeidan *et al*., 2013; Malek and Badran, 2010; Orth and Lutes, 1985). Stable adaptive resistance to phenoxyethanol in both *B. lata* and *B. cenocepacia* strains resulted in 1.5 to 3-fold increases of the MIC, although this still accounted for a small percentage of the regulated permitted amount 15.3 - 23.3% (Table 18). For both the H/F and L/S methodology, the trajectory of resistance was linearly increasing with each pattern and could have been increased further (Figure 28). The development of stable adaptive resistance to phenoxyethanol gained by Bcc strains even under (unfavourable) high concentration gradients, demonstrates that this preservative may not offer a stable long-term preservation strategy. This is also reflected in recent evidence which demonstrated that nosocomial bacterial strains are able to gain adaptive resistance to alcohol-based disinfection strategies. A study investigating isopropanol resistance amongst 139 clinical isolates of *E. faecium* between 1997 and 2015 found that isolates detected after 2010 had a 10-fold higher resistance than older isolates. Strains used in mouse gut infection models were able to survive 70% (v/v) isopropanol treatment, the in-use disinfection concentration (Pidot *et al*., 2018). Therefore, while adaptive resistant trained Bcc strains are still within the current regulated in-use guideline, it demonstrates the potential ability to gain high levels of adaptive resistance in the future.

Stable BIT induced adaptive preservative resistance was gained by both *B. cenocepacia* and *B. lata* strains (Table 17). However, concentration levels remained low, below 3% of the permitted in-use concentration (Table 18). Whilst stability of resistance did not differ by method, Bcc strains evolved using the L/S methodology achieved resistance double that of the H/F methodology, indicating that long exposure to sub-inhibitory concentrations are essential for high resistance. For the L/S methodology, the trajectory of resistance was linearly increasing with each pattern and could have been increased further (Figure 28). Due to their long history of use, adaptive resistance to isothiazolinone preservatives by bacteria has been well documented in prolific industrial-contaminant bacterial species such as *Burkholderia* species, *Pseudomonas* species, *Escherichia* species, *Aureobacterium* species, *Enterbacter* species and *Bacillus* species (Périamé *et al*., 2015; Cloete, 2003; Winder *et al*., 2000; Brözel *et al*., 1995; Brözel and Cloete, 1994; Collier *et al*., 1990; Orth and Lutes, 1985; Wallhausser, 1984). *Burkholderia* species can adapt to high concentrations of BIT preservatives, and product contaminant strains have been described as displaying 4 to 9-fold increases (Rushton *et al*., 2013; Chapman *et al*., 1998). Therefore, despite synthetic preservatives such as BIT having a lower potential resistance, there is the potential for high level adaptive resistance to be gained .

Variation in adaptive resistance potential can be seen within the *B. cenocepacia* group. *B. cenocepacia* strain 1318 demonstrated a lower adaption potential to alcohol-based nature identical preservatives than *B. cenocepacia* strains 1291 and 1292 (Table 17). During L/S adaptive resistance experiments with benzyl alcohol *B. cenocepacia* strains 1291 and 1292 reached MIC points, however for *B. cenocepacia* 1318 preservative sensitivity increased and viability decreased (Figure 28). *B. cenocepacia* 1318 demonstrated stable adaptive preservative resistance to phenoxyethanol (1.5-fold) but this was modest compared to other strains *B. cenocepacia* (2-3fold). Therefore, adaptive resistance potential is not correlated by species type.

Cross resistance of biocides and preservatives to other antimicrobials and physiological stresses has been seen in micro-organisms and is thought to due in part through the activation of general resistance mechanisms (Davin-Regli and Pages,

2012). Cross resistance of stable preservative adapted *Burkholderia* strains was not tested however *B. lata* strain 1299 displayed a 10 times greater innate resistance to BIT than other *Burkholderia* strain. This strain also exhibited a transient increase in susceptibility to benzyl alcohol preservative which was not demonstrated by any *B. cenocepacia* strains (Table 17). However, the MIC profile of *B. lata* strain 1299 to benzyl alcohol and phenoxyethanol preservatives was on the lower end of the scale than other stains (Figure 27). Therefore, although 2-3 fold increases in adaptive resistance to benzyl alcohol and phenoxyethanol was seen, this was often due to low initial MIC's, and did not exceed MIC points observed in *B. cenocepacia* strains. It can be concluded that the intrinsically BIT resistant *B. lata* strain 1299 did not exhibit cross resistance to other preservative agents. A previous experiment looking at the preservative susceptibility profiles of preservative-adapted *B. lata* strains found cross resistance to other preservatives and antibiotics varied considerably demonstrating mechanisms of adaptive resistance may be agent and/or strain specific (Rushton *et al*., 2013).

4.4.2 Testing Resistance Stability

Stable adaptive resistance is defined as long term stable increase above MIC values that persist even without antimicrobial treatment and transient adaptive resistance is defined as short term increases above MIC that return to wild type levels in the absence of antimicrobial (Fernández *et al*., 2011). According to current theory, fast adaptive resistance methodology should encourage a transient response while slower methodologies should induce a stable adaptive resistance response (Motta and Aldana, 2016). In this study, two methodologies were designed: A high and fast (H/F) protocol using the highest concentrations of preservative in fast successive passages (24-48 hours) and the low and slow (L/S) protocol using sub-inhibitory concentrations in slow passages (24-72 hours). Stability of increased resistance phenotypes in the absence of the priming preservative was examined. Retainment or reversion of preservative resistant phenotypes was varied and did not correspond to protocol type which suggests stability of adaptive resistance is primarily powered by strain and agent factors (Table 17).

Stable adaptive resistance was seen with the preservation agent phenoxyethanol, across all four Bcc strains regardless of methodology (Table 17). This suggests that resistance to phenoxyethanol is agent specific and is not transient in nature.

Species specific stability is indicated with the preservative agent BIT, where *B. cenocepacia* strains retained stable increases gained in both H/F and L/S experiments, while the resistant phenotype gained by *B. lata* strain 1299 in L/S experiments was lost in absence of the preservative (Table 17). Stability of adaptive resistance responses is not corelated with level of resistance as modest 1.5-fold MIC increases observed with *B. cenocepacia* species were stable, whilst increases of the same level resulted in *B. lata* strain 1299 reverted back to MIC levels.

4.5 Conclusion

Investigation of preservative susceptibility of a collection of 47 *Burkholderia* isolates demonstrated that susceptibility was varied across strains and agents (even of the same class). Some strains exhibited exceptional resistance to select preservatives, close to the maximum permitted in-use concentration.

Nature identical preservatives salicylic acid demonstrated in use concentration lower than the synthetic preservatives DMDMH and methyl paraben. benzoic acid preservatives required high in use concentrations to demonstrate efficacy, but this was comparable or lower to the synthetic preservative DMDMH and marginally higher than methyl paraben. Nature identical preservatives required significantly higher concentration to effectively inhibit *Burkholderia* isolates than isothiazolinone synthetic preservatives. The microbial activity of the organic acid class of nature identical preservatives (benzoic acid and salicylic acid) varied and MIC's with these agents lacked consistency.

Stable adaptive resistance was developed for *B. cenocepacia* strains to the synthetic preservative BIT under long exposure timeframes and multiple low concentrations gradients. Stable adaptive resistance to the nature identical preservative Phenoxyethanol was developed with *B. cenocepacia* and *B. lata* strains with both short/long timeframes and high/low concentration gradients. Stable adaptive resistance to Benzyl alcohol preservative could not be developed with *B. cenocepacia* and *B. lata* strains.

A link between duration of contact with antimicrobial and/or concentration of sub-inhibitory exposure and stability of adaptive preservative resistance could not be demonstrated.

Chapter 5: Genomic Analysis of Preservative Adaptive Resistance in Bcc Strains

5.1 Introduction

5.1.1 Biocide Resistance

Preservative resistance is not new, and whilst the first report of biocide resistance was cited in 1896 the manufacturing industries awareness predates this by decades (Orth and Lutes, 1985; Heathman *et al*., 1936). Unlike antibiotic resistance research, academic research on biocide resistance is sparse and usually centres around environmental/product contamination events or small-scale lab controlled Adaptive Laboratory Evolutions (ALE) (Maillard, 2018). Reports of sporadic contamination events are often reported from an infection control perspective and focus on infections, recalls and level of resistance while rarely acknowledging the root cause such as improper in-use practices, preservative failure or preservative resistance (Weber *et al*., 2007). The applicability of ALE experiments performed *in vitro* and in isolation of environmental factors and the product environment has been questioned as these factors have a significant impact on biocide efficacy (Bock *et al*., 2016; Cowley *et al*., 2015; Maillard and Denyer, 2009; SCENIHR, 2009; Walsh *et al*., 2003).

Unlike antibiotic resistance research, terminology for biocide resistance is vague. Some studies define biocide resistance in microbiological resistance terms with resistance measured against a previously observed MIC (Maillard *et al*., 2013; Maillard, 2007; Thomas *et al*., 2000, 2005; Walsh *et al*., 2003; Malek *et al*., 2002; Tattawasart *et al*., 1999). MIC changes are a good trend indicator for resistance development and have been widely used to study bacterial biocide resistance mechanisms (Wesgate *et al*., 2016; Alonso-Calleja *et al*., 2015; Curiao *et al*., 2015; Knapp *et al*., 2015; McMurry *et al*., 1999; McMurry *et al*., 1998). Other studies utilse the in-use concentration or MBC much like a clinical “breakpoint” and are only concerned with resistance above this threshold (Lerma *et al*., 2012; Cerf *et al*., 2009). While useful for bactericidal disinfectants and antiseptics the MBC is less applicable in terms of preservation as products are only meant to be bacteriostatic (Knapp *et al*., 2015; Maillard *et al*., 2013; Maillard, 2007).

The lack of standardised reporting practices compounds the ability to accurately cross compare and correlate biocide resistance patterns (Maillard, 2018; Maillard *et al*., 2013). Biocide resistance (in the environment and *in situ*) has been widely reported against several biocides classes including Bisbiguandies (e.g., chlorohexidine) (Gadea *et al*., 2017; Thomas *et al*., 2000, 2005; Tattawasart *et al*., 1999; Kurihara *et al*., 1993), quaternary ammonium compounds (QACs) (e.g., benzalkonium chloride) (Wickham, 2017; Ahn *et al*., 2016; Latimer *et al*., 2015; Bragg *et al*., 2014; Guo *et al*., 2013; Joynson *et al*., 2002; Kurihara *et al*., 1993; Nishikawa *et al*., 1979; Chaplin, 1951), Phenolics (Triclosan) (Forbes *et al*., 2015; Latimer *et al*., 2012; Walsh *et al*., 2003; McMurry *et al*., 1999; McMurry *et al*., 1998), Isothiazolinones (Green *et al*., 2018; Rushton *et al*., 2013; Winder *et al*., 2000; Sondossi *et al*., 1999; Brözel and Cloete, 1994), Alcohols (Green *et al*., 2018; Gupta *et al*., 2018; Malek and Badran, 2010) and Parabens (Selvaraj *et al*., 2013; Flores *et al*., 1997; O'Neill and Mead, 1982).

With the of rise and dissemination of antibiotic resistance, some studies have also highlighted worrying trends in biocidal resistance. Longitudinal studies of environmental nosocomial pathogens have shown that strains isolated decades earlier are more susceptible to commonly used biocides than current isolates. An *in-situ* study using *S. epidermidis* isolates cultured prior to widespread use of Triclosan found all 34 isolates were susceptible to 0.25 mg/L Triclosan while 8 of the 64 current *S. epidermidis* strains exhibited tolerance. Furthermore, in ALE experiments, all isolates could gain resistance to Triclosan, but for the majority of current *S. epidermidis* isolates this was not attributed to mutations associated fabI or associated promoter regions (Skovgaard *et al*., 2013). Another study of *E. faecium* nosocomial isolates obtained between 1997 and 2015 found isolates cultured after 2010 exhibited a 10-fold higher tolerance to 70% isopropanol disinfectant. Comparative genomic analysis of susceptible and alcohol tolerant *E. faecium* found mutations in genes controlling metabolism. Mutagenesis of these genes resulted in regained susceptibility in alcohol tolerant *E. faecium* (Pidot *et al*., 2018). While the studies are limited to isolates found in single geographic locations (and are not a comment on the bacterial population as a whole), the temporal shift in biocide tolerance for isolated nosocomial pathogens indicates increasing biocide resistance.

5.1.2 *Burkholderia* Genomes

Bioinformatics analysis regarding Bcc strains is challenging due to the lack of annotated whole genome information. At present, according to the NCBI Genome resource there are 18 complete genomes for the prominent Cystic Fibrosis associated pathogen *B. cenocepacia*, and only 3 for *B. lata*. Even with reference genomes, variation is seen within *Burkholderia* strains and MLST types. One study found when aligning 11 Bcc outbreak strains to reference genomes, 31.6–48.3% could not be aligned resulting in 1123–2139 false single-nucleotide polymorphisms (SNPs) (Abdelbary *et al*., 2018). Therefore, the analysis of whole genome *Burkholderia* contaminant strains is essential for the molecular characterisation of preservative resistance mechanisms.

5.1.3 Classical Resistance Mechanisms

Much of the research on biocide resistance has followed research on antibiotic resistance. However, the differences between antibiotics and biocides in their targets, mode of action, and intended outcome are widely different (Wales and Davies, 2015). Antibiotics are single in their target and therefore very simplistic in their mode of action, whilst the intended outcome is to result in bactericide, clinical doses have to ensure a lack of toxicity to the patient. Biocides however are multi-target, with their mode of action affecting multiple cell processes and components and the in-use concentrations are generated to produce maximum lethality, with a consideration of sensitisation/toxicity effects.

Current theory on antibiotic resistance assumes that resistance arises from multiple mechanisms including modification of target site, increase in target protection, enzymatic inactivation, reduced cellular permeability and activation of efflux systems (Munita and Arias, 2016). Mechanisms of biocide action are poorly understood and are dependent on the biocide, the method of application and the strain/species (Wales and Davies, 2015). Current scientific evidence on biocide resistance suggests that the emergence of general bacterial biocide resistance is not determined by a single trait but instead is a co-ordination of various elements of the bacterial biocide resistome. Several mechanisms have been described in biocide resistance including induction of active efflux, reduction in cellular permeability, enzymatic degradation and biofilm formation (Coenye and Mahenthiralingam,

2014). Due to their multitarget mode of action, biocide resistance is therefore unlikely to be caused by a specific modification of a target site or in target protection (SCENIHR, 2009).

There is a distinction between types of antimicrobial resistance. Intrinsic antimicrobial resistance is described as the natural antimicrobial properties of an organism. Acquired resistance is the consequence of the acquisition of genetic elements (e.g. horizontal gene transfer and plasmids). Adaptive resistance is the sub-inhibitory exposure of antimicrobials to an organism which results in an induction/mutation within resistance mechanisms which results in a previously susceptible bacterial population obtaining increased antimicrobial resistance (Motta and Aldana, 2016). This is reliant on sub-inhibitory exposure and therefore is separate from intrinsic and acquired resistance which do not rely on antimicrobial selective pressure. In clinical settings antimicrobial resistance occurs most commonly via adaptive resistance mechanisms (Munita and Arias, 2016). This is further complicated with the identification of stable adaptive resistance mechanisms, whereby the baseline susceptibility does not revert back once the antimicrobial is removed. There is a large cross over between intrinsic and adaptive mechanisms with shared mechanisms of enzymatic degradation, porin modification, outer membrane permeability and efflux activity. In transcriptomic analysis of *Burkholderia* resistance to antimicrobial agents, known resistance elements often fail to identify in gene expression studies due to the high innate expression of resistance elements such as efflux (Sass *et al*., 2014; Yang and Speed, 2002) . This indicates that identifying mechanisms of adaptive resistance may be difficult to distinguish from innate mechanisms of antimicrobial resistance.

5.1.3.1 Enzymatic Degradation

Many gram-negative bacteria, most notably alpha, beta and delta proteobacteria have diverse metabolic capacities and can degrade a multitude of toxic aromatic compounds. Environmental bio-degradation has been well described by soil microbial *Burkholderia* species. These strains are able to mineralise xenobiotics into harmless by-products which has attracted interest for environmental application in the area of bioremediation (Valderrama *et al* 2012). Various studies in *Burkholderia* species have demonstrated the induction of enzymatic bio-degradation enzymes and

pathways in response to toxic compounds including aromatic compound degradation (Denef *et al*., 2006), polychlorinated biphenyls (PCBs) (Agulló *et al*., 2007), heavy metals (Van Nostrand *et al*., 2008) and herbicides (Li *et al*., 2009) Interestingly, in *Burkholderia* species toxic compound resistance is also seen to involve resistance to physiological stress and vice versa (Battesti *et al*., 2011).

Several *Burkholderia* species have demonstrated enzymatic degradation-based resistance mechanisms to beta lactam and aminoglycoside antibiotics. There may also be a link between antibiotic and solvent enzymatic breakdown. A recent soil metagenomic study which screened beta-lactam resistant libraries also found a novel group of dioxygenases which were capable of breaking down aromatic compounds (dos Santos *et al*., 2015). At present little is known about the mechanisms and significance of enzymatic degradation as a bacterial biocide resistance mechanism (SCENIHR, 2009). In bacteria the enzyme mediated degradation of various biocides has been described for metallic salts (Cloete, 2003), parabens (Valkova *et al*., 2002), aldehydes (Kümmerle *et al*., 1996), quaternary ammonium compounds (QACs) (Nishihara *et al*., 2000) and phenols such as triclosan (Hundt *et al*., 2000).

5.1.3.2 Reduced Cellular Permeability

Changes in cellular permeability physically restrict biocide penetration into the cell, thereby reducing intracellular biocide disruption. In gram-negative bacteria the double membrane cellular structure enables greater control and modification of antimicrobial substances (Denyer and Maillard, 2002). The two main methods of cellular permeability resistance are outer membrane changes and changes to outer membrane porins. Biocidal activity is observed to be reduced through the action of non-specific outer membrane structure changes (Braoudaki and Hilton, 2004; Tattawasart *et al*., 1999, 2000) and specific changes in phospholipid composition (Heredia *et al*., 2014; Boeris *et al*., 2007) and fatty acid composition (Guérin-Méchin *et al*., 2004; Dubois-Brissonnet *et al*., 2001; Guérin-Méchin *et al*., 1999). Outer membrane porins are responsible for molecule movement across the membrane and are essential for cellular permeability(Denyer and Maillard, 2002). Expression or repression of specific porins has been associated with resistance to both antibiotics and biocides (Frenzel *et al*., 2011; Karatzas *et al*., 2008; Malek *et al*., 2002).

5.1.3.3 Efflux Pump Systems

Membrane imbedded efflux pumps are induced in antimicrobial resistant organisms to expel toxic compounds from the cell. The majority of antimicrobial efflux pumps are non-specific and can expel various toxic chemicals, solvents, metals as well as antibiotics, biocides and preservatives (Li *et al*., 2016). The five major classes of efflux pumps are found across all prokaryotes and of these the tripartite efflux pumps of the RND type are the most common. RND type efflux pumps consist of three components: a transporter protein located in the inner-membrane, a membrane adaptor protein (MFP) in the periplasm and an outer membrane exit channel protein (OMP) (Du *et al*., 2018). There is a strong link between efflux pumps and antimicrobial resistance as demonstrated through increased antimicrobial susceptibility with inactivation of efflux pumps and the addition of efflux pump inhibitors. Furthermore, the overexpression of efflux pumps, activation of efflux pump regulators and increases in efflux activity have been seen to result in increased antimicrobial resistance (Wales and Davies, 2015). In RND knock outs of *B. cenocepacia* J2315 efflux pumps are also seen to be involved in additional traits such as biofilm synthesis, chemotaxis driven motility and flagellum expression. This is thought to be through the action of intracellular efflux of homoserine lactone signalling molecules (Bazzini *et al*., 2011).

5.1.3.4 Biofilm

Biofilm growth modes are a known antimicrobial resistance strategy that afford significant protection to a bacterial population (Chapman, 2003). *Burkholderia* biofilms have been studied most intensively in terms of clinical CF infections, where during chronic infection a movement to mucoid phenotype is observed (Zlosnik *et al*., 2014). *Burkholderia* biofilm colonisation in CF patients often results in prolonged chronic infection due to their high resistance to antibiotics, making them difficult to eradicate (Kennedy *et al*., 2016; Dales *et al*., 2009; Caraher *et al*., 2007). Biofilm formation results in limited biocide diffusion into the matrix structure (Szomolay, 2008) and limited effect due to dormancy state (Rani *et al*., 2007).

5.1.4 Other Factors of Resistance

The resistome is incredibly broad and includes both classical resistance genes and also vaguer genes that may seemingly contribute little resistance. Recent research

has looked into a broad array of contributing antimicrobial resistance factors including metabolic state and activity, SOS response, ROS response, as well as physiological stress factors such as heat/cold shock. While these elements of resistance are non-specific, they seem to play a role in bacterial antimicrobial resistance (Poole, 2014).

5.1.4.1 Stress Resistance and Damage Response

The metabolic capabilities of prokaryotes enable survival of physical damage from environmental extremes such as high and low pH's, environments with high metal compound levels, high salinity and osmolarity, desiccation, exposure to ultraviolet radiation, high and low temperatures and variable oxygen availability (Atashgahi *et al*., 2018). *Burkholderia* species have shown exceptional ability to survive and thrive in a variety of adverse environments including human, animal and plant hosts. They can tolerate an array of physical and chemical stresses such as heat, high osmolarity, detergents, heavy metals, ROS, biocides and antibiotics (Coenye and Mahenthiralingam, 2014). Exposure to oxidative agents in *B. cenocepacia* J2315 is seen to activate a multitude of responses including induction of detoxifying (Peeters *et al*., 2010), damage repair mechanisms and DNA polymerases (Kang *et al*., 2011; Sass *et al*., 2011). Stress response mechanisms affect bacterial antimicrobial susceptibility in various ways. Research suggests that in early exposure to antibiotics, stress responses are essential to mediate antibiotic stress (Eswara and Kumavath, 2017).

5.1.4.2 Metabolism

The metabolic state of bacterial populations can change susceptibility to antimicrobial agents. Bacterial persistence is where a sub-population enters a dormant state. As antibiotics on work on metabolically active cells the arrested growth rate and limited metabolic state of persister subpopulations result in phenotypic resistance to antibiotics (Cohen *et al*., 2013).

Pervious research focused on the specific mechanisms of antimicrobial resistance and the effect of resistance on bacterial metabolism was defined as a 'fitness' loss. However, new scientific research utilising proteomics and metabolomics indicate that metabolism may be directly involved in antimicrobial resistance. In the

proteomic analysis of an *E. coli* strain that was 64 times more resistant to piperacillin/tazobactam than wild type found that glycolysis and energy metabolism enzymes were highly abundant in resistant strains compared to wild type (dos Santos *et al*., 2010). Biocides are also seen to affect various metabolic processes. Triclosan-resistant mutants of *E. coli* and *K. pneumoniae* demonstrated increased metabolism of carboxylic acids, amino acids, and carbohydrates (Curiao *et al*., 2015).

In summary, Bcc species are highly resistant to antimicrobials including commonly used home and personal care preservatives. This has major implication on product preservation systems and industrial decontamination. Understanding the underlying mechanisms of preservative resistance is important for improving preservation strategies and preventing resistance in Bcc populations. Little is known about the preservative resistome and cases of preservative resistance are on the rise. More information is needed regarding the molecular mechanisms of resistance for common industrial contaminants such as the Bcc.

5.2 Aims

The aims of this chapter were as follows:

1. To examine the abundance of gene affecting variants within *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318 that were grown in progressive subculture at low concentration gradients and slow timeframes or high concentration gradients and fast timeframes with BIT, benzyl alcohol and phenoxyethanol preservative agents which displayed transient and stable preservative resistant phenotypes.

2. To investigate the effect of subculture method type, strain type and preservative type on the presence and putative function of gene affecting variants within *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318 that displayed stable preservative resistant phenotypes.

3. To examine the putative function of gene affecting variants within *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318 that displayed stable preservative resistant phenotypes.

4. To investigate the effect of subculture method type, strain type and preservative type on the presence and putative function of gene affecting variants within *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318 that were grown in progressive subculture with preservative agents.

5. To examine the putative function of gene affecting variants and GO enrichment profiles within *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318 that were grown in progressive subculture with preservative agents.

5.3 Methods

5.3.1 Preparation of DNA for sequencing

5.3.1.1 Growth and DNA Extraction of *B. lata* and *B. cenocepacia* Strains from Adaptive Resistance Experiments

Timepoint 0 and termination timepoint -80°C stocked cultures from evolution experiments (see chapter 4) were spread onto BSM agar without preservative. Plates were incubated for 48 hours at 30°C. Sterile universals containing 3 ml of BSM broth with the termination concentration of preservative were inoculated and incubated for 18 hours at 30°C (Table 19). Cells were washed in sterile PBS three times before DNA extraction using the Bioline ISOLATE II Genomic DNA kit (London, UK). The protocol was followed as per the manufacturer's instructions, except for the duplication of Wash buffer GW2 step. Due to the presence of impurities, presumably from co-extracted preservative, DNA was purified using New England Biolabs AMPure XP Beads (Ipswich, US) according to the manufacturer's instructions.

Table 19: Termination concentrations for preservative adaptive resistance experiments

Terminal Concentration % (v/v)					
Preservative	**Method Type**	***B. cenocepacia***			***B. lata***
		1291	**1292**	**1318**	**1299**
Benzyl Alcohol	H/F	0.153	0.153	0.109	0.109
	L/S	0.206	0.219	0.206	0.187
BIT	H/F	0.00123	0.00123	0.00125	0.0049
	L/S	0.00249	0.00249	0.00312	0.00973
Phenoxyethanol	H/F	0.153	0.153	0.153	0.153
	L/S	0.267	0.237	0.160	0.237

5.3.1.2 Illumina Library Preparation and Sequencing Protocol

Library preparation was performed by the Centre for Genomic Research (CGR), Liverpool. The method was as follows: DNA at a concentration of 200 ng was sheared with the Diagneode Picoruptor (Liege, Belgium) to generate fragments approximately 550bp. These were cleaned with AMPure beads (New England

Biolabs, Ipswich, US) and end repaired. Following the end repair reaction, the sample was size selected with the sample prep beads. The samples were A tailed and adapter ligated. Libraries were cleaned with equal volume of AMPure beads and checked with Invitrogen's Qubit fluorometric assay (ThermoFisher Scientific, Waltham US) and Agilent Bioanalyser chip (Santa Clara, US). Samples were pooled with equal representation of each sample based on QPCR and sequenced (2 x 300bp) on a single lane of the Illumina MiSeq platform.

The generated fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 using the option -O 3 so the 3' end of any reads which match the adapter sequence for 3 bp or more were trimmed. The reads were further trimmed using Sickle version 1.200 with a minimum window quality score of 20. Reads shorter than 10 bp after trimming were removed.

5.3.1.3 Growth and DNA Extraction of *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318

A inoculation loop was used to spread -80°C stocks onto BSM agar plates and plates were incubated for 12 hours at 30°C . Sterile universals containing 3 ml of BSM broth were inoculated and incubated for 18 hours at 30°C. Cells from 0.5 ml of from overnight culture were aliquoted into sterile 2 ml Eppendorf tubes and harvested by centrifugation at 8000 rpm at 4°C for 15 minutes. The supernatant was removed, and the pellet was washed twice in sterile PBS and centrifuged at 8,000 rpm at 4°C for 15 minutes. Cells were lysed by adding 50 µl of 10 mM Tris-HCl (pH 8) and 2.5 mg/ml of freshly prepared lysozyme and incubated at 37 °C for 1 hour. Cells were treated with 100 µl of lysis buffer (50 mM Tris, 100 mM EDTA, 1% SDS, pH 8) and 10 µl of 20 mg/ml of Proteinase-K and incubated at 55°C for 1 hour. 800 µl of cooled lysed sample and 800 µl of room temperature phenol chloroform isoamyl alcohol (25:24:1) were mixed gently for 5 minutes and centrifuged at 10,000 rpm 4°C for 10 minutes. The upper layer was transferred to a fresh sterile tube and mixed with an equal volume of chloroform isoamyl alcohol (24:1) and centrifuged at 10,000 rpm 4°C for 5 minutes. The upper layer was removed and transfer to a new tube. To this a 1/10 ratio of 3M sodium acetate (pH 5.2) was added and mixed well. This was followed by adding a 2.5 volume of ice-cold molecular grade 100% ethanol and mixed gently then incubated at -20°C overnight. This was spun for at 10,000 rpm 4°C for 20 minutes. The supernatant was removed, and the pellet was washed in

300 µl of 80% ethanol and vortexed gently 3 times and then centrifuged at 10,000 rpm 4°C for 15 minutes. The supernatant was removed, and the wash step was repeated. The supernatant was removed and spun for 2 minutes at 10,000 rpm 4°C, and all residual ethanol. The pellet was left to air dry for 1-2 minutes and then 100 µl of TB buffer (pH 8) was added and the pellet was left to dissolve for 5-6 hours. This was repeated until 10 µg of gDNA was isolated. gDNA was purified New England Biolabs AMPure XP Beads (Ipswich, US) according to the manufacturer's instructions. DNA quantity and quality were measured using the Nanodrop Spectrophotometer and OD260/280 was between 1.8 and 2.0 and OD260/230 was between 2-0 – 2.2.

5.3.1.4 PacBio Library Preparation and Sequencing Protocol

Library preparation was performed by the Centre for Genomic Research (CGR), Liverpool. The method was as follows: Supplied material was purified with 1x cleaned AMPure beads (New England Biolabs, Ipswich, US) and the quantity and quality was assessed using Nanodrop spectrophotometer and Qubit assay. In addition, Advanced Analyticals Fragment Analyser (Agilent, Santa Clara, US) was used using a high sensitivity genomic kit to determine the average size of the DNA and the extent of degradation. This procedure was also used at the steps indicated below to determine average fragment size of the DNA. DNA was sheared using Covaris (Woburn, US) g-TUBE at 4600 rpm centrifugation speed in an Eppendorf centrifuge. This yielded approximately 10 kb average sized fragments as determined by the Fragment Analyser.

DNA was treated with Exonuclease V11(New England Biolabs, Ipswich, US) at 37 °C for 15 minutes. The ends of the DNA were repaired as described by Pacific Biosciences (Menlo Park, US) protocol. The sample was incubated for 20 minutes at 37 °C with DNA damage repair mix supplied in the SMRTbell library kit (Pacific Biosciences). This was followed by a 5 minute incubation at 25 °C with end repair mix. DNA was cleaned using 0.5x AMPure beads and 70% ethanol washes.

DNA was ligated to adapter overnight at 25 °C. Ligation was terminated by incubation at 65 °C for 10 minutes followed by exonuclease treatment for 1 hour at 37 °C. The SMRTbell library was purified with 0.5x aMPure beads. The library was size selected with 0.75% blue pippin cassettes (Sage Science, Beverly, US) in the range 7000-20000 bp. The recovered fragments were damage repaired again. The

quantity of library and therefore the recovery was determined by Qubit assay and the average fragment size determined by Fragment analyser.
SMRTbell library was annealed to sequencing primer at values predetermined by the Binding Calculator (Pacific Biosciences) and a complex made with the DNA Polymerase (P6/C4 chemistry). The complex was bound to MagBeads (Pacific Biosciences) and this was used to set up the required number of SMRT cells (Pacific Biosciences) for the project. Sequencing was done using 360-minute movie times.

5.3.2 Bioinformatics Analysis

All scripts are given in Appendix 1.

Reference genomes for *B. cenocepacia* and *B. lata* were sequenced using PacBio SMRT Cell long-read sequencing technology. Genomes were assembled in the PacBio SMRT portal using their Hierarchical Genome Assembly Process (HGAP). The polished assembly was produced by the Centre for Genomic Research (CGR), Liverpool. The timepoint 0 and terminal timepoint of each adaptive resistance experiment was sequenced using Illumina MiSeq generated paired end sequencing. To assess quality, the illumina sequencing reads were aligned against the PacBio polished assembly genome references using Bowtie2 Version 2.2.5 (Build 2015-03-06) (Langmead and Salzberg, 2012).

Adaptive resistance sequencing samples with a good overall alignment with the reference, underwent Genome Analysis Tool Kit (GATK) pre-processing protocol as outlined in GATK best practices (Auwera *et al*., 2013; DePristo *et al*., 2011). Raw Illumina fastQ reads were mapped to PacBio polished assembly genome references in BWA version 0.7.12 (Build 2014-12-28) (Li and Durbin, 2009). Raw mapped reads were processed in Picard Tools Version 2.16.0 (Build 5.12.2017) to mark and remove duplicates of mate pair reads that are causative of sequencing processes and not genuine variant alleles (Broad Institute, 2017). Samtools Version 1.3 (Build 2015-12-15) was used to create a GATK compatible BAM (Binary Alignment Map) file sorted on co-ordinates and indexed (Li *et al*., 2009).

GATK haplotype caller (GATK Version 3.7 Build 2016-12-30) was used to identify genomic variation in samples (McKenna *et al*., 2010). Post haplotype calling, joint

genotyping was not utilised as samples of the same species/strain type were grown in different preservatives and therefore were not expected to have variant consensus. GATK VariantsToTable function was used to output significant variant annotations (FisherStrand (FS), QualByDepth (QD), RMSMappingQuality (MQ), MappingQualityRankSumTest (MQRankSum) and ReadPosRankSumTest (ReadPosRankSum). This data was used to create density plots with ggplot2 Version 2.2.1 (Build 2016-12-30) in R to assess hard filtering threshold applicability. Due to low QD values presumably caused by high ploidy, variants were filtered according to the top 10 % of quality (QD) ranked SNPs and Indels. All other quality recommendations were followed. SNPs and Indels were extracted from the VCF files and then variant filtration thresholds were applied to each separately. Annotated VCF files were inputted into snpEFF Version 4.3 (Build 2017-11-24) for additional annotation of functional effect of variants (Cingolani *et al*., 2012). snpSift Version 4.3 (2017-11-24) was used to output variant information into text files (Cingolani *et al*., 2012).

Microsoft Excel Version 16.24 was used to concatenate SNP and Indel text files, remove variants present in both controls and experiments and to remove genes with unknown functions. Experimental samples were assessed for high variant count which indicated contamination, and these were omitted from further analysis. For experiments which demonstrated stable adaptive resistance Microsoft Excel was used to further filter out any genes shared with experiments which did not demonstrate resistance or demonstrated transient resistance. Variant function analysis was undertaken for experiments which demonstrated stable adaptive resistance and for all strains exposed to sub-inhibitory concentrations of preservatives, regardless of whether a change in preservative resistance was exhibited or retained. Genes of interest were identified as present in more than one experiment and therefore had a higher likelihood of being selected rather than the product of random mutagenesis.

GO Enrichment Analysis was performed in R version 3.4.0 (build 2017-04-21) using the topGO package (Version 2.24.0, build 2017-04-24) (Alexa *et al*., 2006). The read mapping for the four PacBio polished assembly genomes was produced using Blast2Go Version 1.3.3 (build 11/11/2016) (Götz *et al*., 2008). The input of a gene

list with gene scores was created manually through concatenating experimental and control data to provide a list of genes containing SNPs/Indels with experimental genes assigned a value of "0" to show significance while control genes were valued at "1" to signify these were not significant. Each experimental sample was run in topGO with three different ontologies: Molecular Function (MF), Biological Process (BP) and Cellular Component (CC). Node size was set at 5, therefore only groups of genes of 5 and above were included. Due to the lack of gene scores/gene expression information enrichment tests were conducted on gene counts using the fishers exact statistical test. The fishers exact statistical test was used in conjunction with four algorithms "Classic", "Elim", "Weight" and "Weight01" which differentially assess the database and therefore calculate significance differently (Alexa *et al*., 2006). Statistical analysis was outputted to a table and significant GO terms were inputted to the gene ontology consortium website for detailed analysis of function (http://www.geneontology.org/).

5.4 Results

5.4.1 *Burkholderia* Genomes and Mapping

The four Bcc genomes were sequenced using long read sequencing technology. This resulted in deep coverage high quality reference genomes for the basis of bioinformatic analysis. PacBio assembly statistics are given in Table 20.

Table 20: **PacBio sequencing and genome assembly statistics for four *Burkholderia cepacia* complex strains**

Species and Strain Type	Size (Mbp)	Contigs	N50 Contig Length (Mb)	N95 Contig Length (Mb)
B. cenocepacia 1291	8.510515	5	3,042,399	**1,228,169**
B. cenocepacia 1292	8.523121	6	3,049,771	**1,224,270**
B. lata 1299	9.201248	7	2,490,336	**1,187,413**
B. cenocepacia 1318	8.429403	5	2,934,806	**1,275,762**

To check the quality of illumina sequencing data, these samples were aligned with Bowtie2 against reference genome assemblies. Of the 37 samples, 35 had a high total alignment percentage with the reference >88.32% (Table 21). However, two samples, H/F 1299 Phenoxyethanol and L/S 1318 Benzyl Alcohol had poor total alignment percentages (47.31% and 45.07%). This was presumably caused by contamination. Therefore, these samples were removed from further bioinformatic processing as to not affect the results.

Table 21: Illumina sequencing alignment statistics for Bcc strains 1291, 1292, 1299 and 1318 before and after preservative evolution experiments when aligned against PacificBiosciences sequenced reference genomes.

Experiment Type	Strain Type	Preservative	Overall alignment rate (%)
H/F	1291	Phenoxyethanol	99
H/F	1292	Phenoxyethanol	99.15
H/F	1299	Phenoxyethanol	47.31
H/F	1318	Phenoxyethanol	99.23
H/F	1291	BIT	99.55
H/F	1292	BIT	99.54
H/F	1299	BIT	99.5
H/F	1318	BIT	99.18
H/F	1291	Benzyl Alcohol	98.99
H/F	1292	Benzyl Alcohol	98.73
H/F	1299	Benzyl Alcohol	99.39
H/F	1318	Benzyl Alcohol	99.01
L/S	1291	Phenoxyethanol	88.32
L/S	1292	Phenoxyethanol	97.16
L/S	1299	Phenoxyethanol	92.49
L/S	1318	Phenoxyethanol	90.27
L/S	1291	BIT	97.76
L/S	1292	BIT	99.19
L/S	1299	BIT	99.52
L/S	1318	BIT	97.86
L/S	1291	Benzyl Alcohol	99.14
L/S	1292	Benzyl Alcohol	99.5
L/S	1299	Benzyl Alcohol	98.62
L/S	1318	Benzyl Alcohol	45.07
Control: H/F Phenoxyethanol	1291	N/A	99.08
Control: H/F Phenoxyethanol	1292	N/A	99.15
Control: H/F Phenoxyethanol	1299	N/A	99.42
Control: H/F Phenoxyethanol	1318	N/A	99.31
Control: H/F BIT and Benzyl Alcohol	1291	N/A	99.45
Control: H/F BIT and Benzyl Alcohol	1292	N/A	99.07
Control: H/F BIT and Benzyl Alcohol	1299	N/A	99.5
Control: H/F BIT and Benzyl Alcohol	1318	N/A	99.33
Control: L/S BIT	1299	N/A	99.48
Control: L/S Phenoxyethanol, BIT and Benzyl Alcohol	1291	N/A	95.43
Control: L/S Phenoxyethanol, BIT and Benzyl Alcohol	1292	N/A	97.7
Control: L/S Phenoxyethanol and Benzyl Alcohol	1299	N/A	97.76
Control: L/S Phenoxyethanol, BIT and Benzyl Alcohol	1318	N/A	98.64

5.4.2 Variant Statistics

Experimental and control samples (37 in total) were put through the GATK haplotype caller pipeline and the GATK Hard Filtering pipeline.

Variant counts for H/F experiments were often lower than the variant count of controls (see figure 29). A paired t-test was conducted to compare variant abundance in H/F controls (N = 11) and H/F experiments (N=11). There was no statistical difference in variant count observed in H/F controls (M= 245.00, SD= 367.52) and H/F experiments (M= 134.27, SD= 134.27); t(10)= 0.9934, p= 0.3439.

Variant counts for L/S experiments were often higher than the variant count of controls (see Figure 30). A paired t-test was conducted to compare variant abundance in L/S controls (N = 11) and L/S experiments (N=11). There was no statistical difference in variant count observed in L/S controls (M= 8560.18, SD= 26117.53) and L/S experiments (M= 255.45, SD= 288.68); t(20)= 1.0545, p= 0.3042.

One experiment, L/S phenoxyethanol strain 1318 had the greatest count of variants with 87275, which is 31.9 times higher than the highest variant count in any other L/S experiment. This could be erroneous and due to poor sequencing mapping. The mean variants in L/S experiments without this sample (M=688.70) is still 2.5 times higher than the L/S controls, (M=270.70) but this difference is not statistically significant (Paired t-test, t(9)= 1.4372, p= 0.1845). This demonstrates that selection pressure is an important factor in micro-evolution experiments, whereby variant frequency can be used as a proxy for genomic evolution.

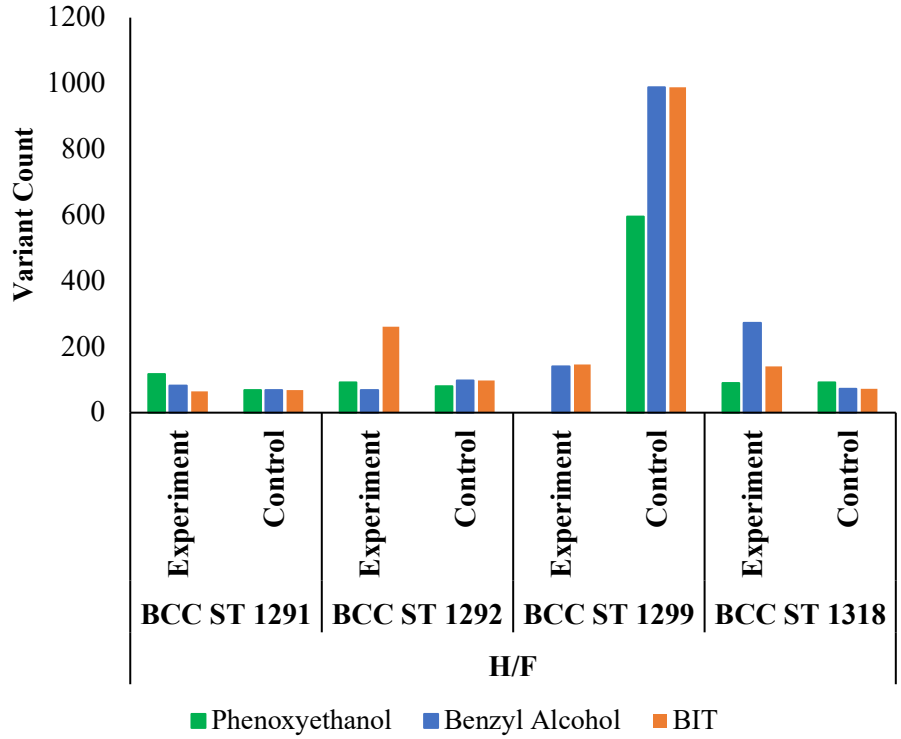


Figure 29: Variant counts for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using high preservative concentrations and fast timescales (H/F)

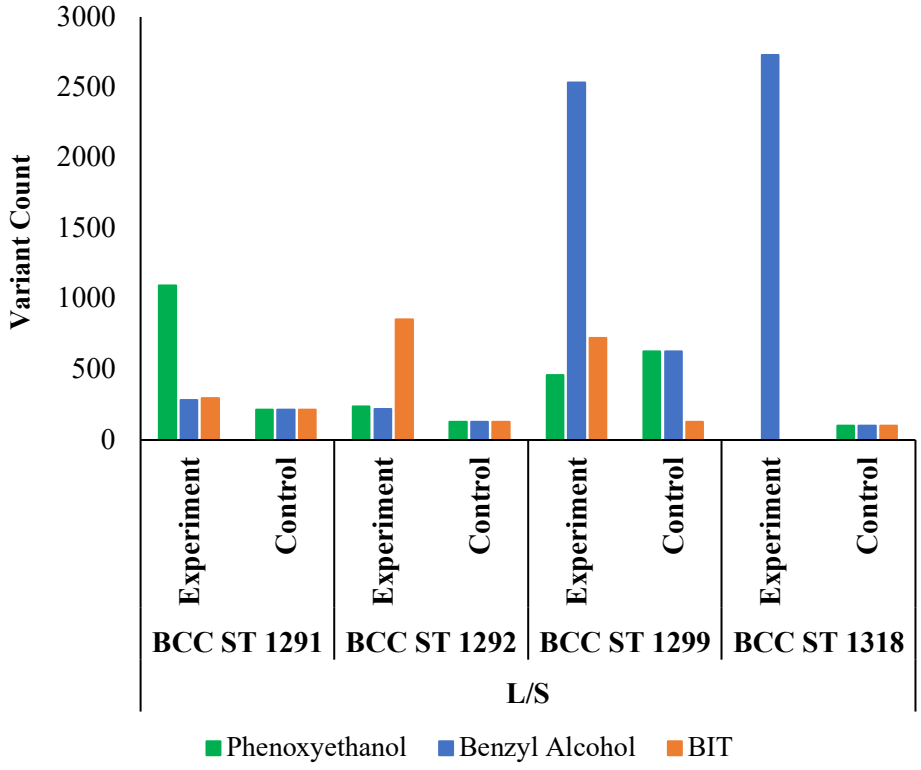


Figure 30: Variant counts for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using low preservative concentrations and slow timescales (L/S). Due to the large variant count in L/S phenoxyethanol strain 1318, this data was omitted.

Due to the high number of variants, data for L/S phenoxyethanol with *B. cenocepacia* strain 1318 (N= 87275) and L/S benzyl alcohol with *B. lata* strain 1299 (N = 2357) were omitted from further analysis. Additionally, due to the large volume of control variants, experimental data was further filtered for removal of variants also found in controls. The total number of variants before filtering, after filtering and the unique experimental variants not found in controls are given in appendix 2.

In assessment of the variant count for each sample (without controls), there was no correlation to fold changes in preservative susceptibility, preservative type (Figure 31A) and species/strain type (Figure 31B). A t-test was conducted to compare variant abundance in Bcc strains which demonstrated a fold change in preservative resistance (N=13) and Bcc strains which retained their wild type preservative susceptibility (N=7). There was no statistical difference for the variant count for Bcc strains which demonstrated an increase in preservative resistance (M= 86.54, SD= 107.16) and those that did not (M= 48.57, SD= 30.67); t(18)= 0.9072, p= 0.3763). Similarly, a t-test was conducted to compare variant abundance in Bcc strains which demonstrated a stable adaptive preservative resistance (N=12) and strains that demonstrated transient or no change in preservative resistance (N=8) however there was no statistical difference in the variant count for Bcc strains which demonstrated a stable increase in preservative resistance (M= 79.92, SD= 109.12) and those that did not (M= 63.25, SD= 50.30); t(18)= 0.4018, p= 0.6926.

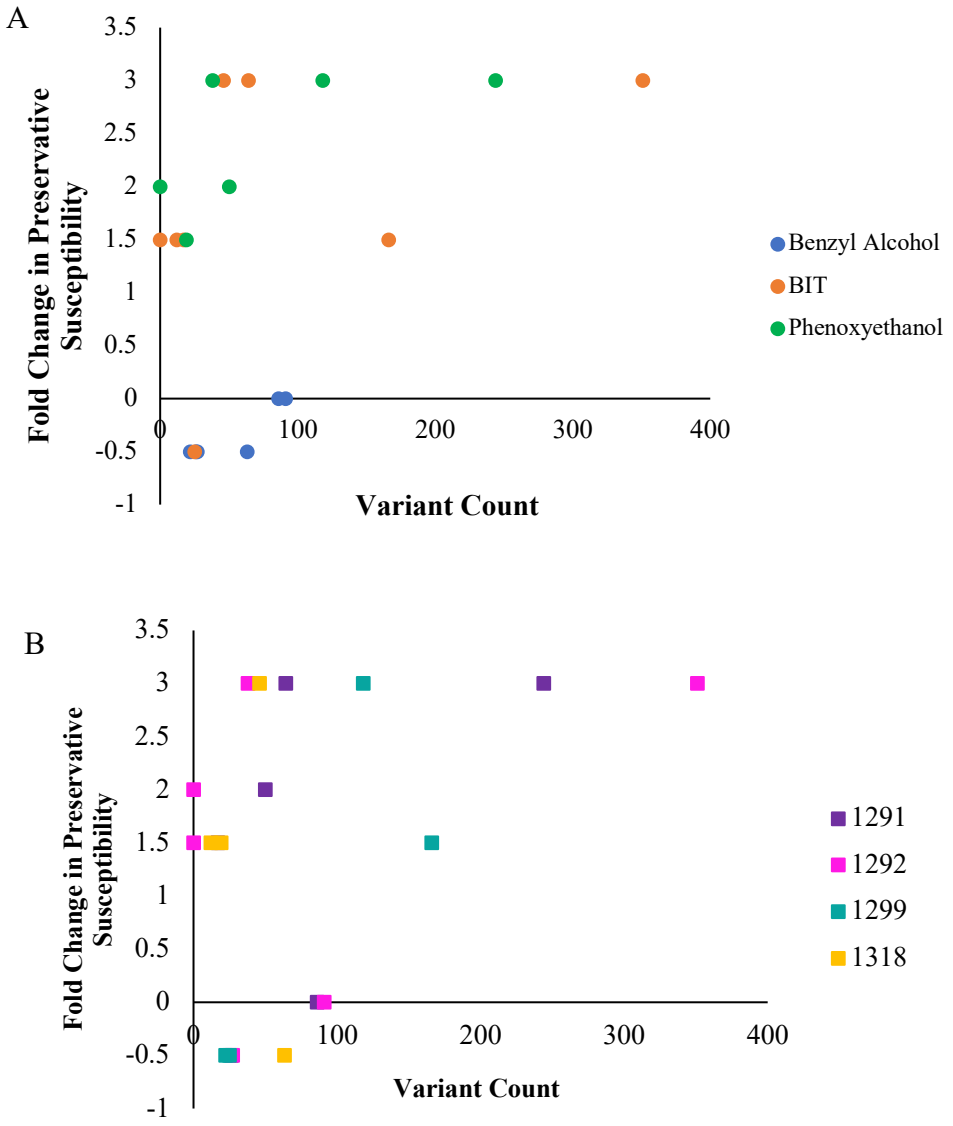


Figure 31: Variant counts (SNP and Indel) for Bcc strains 1291, 1292, 1299 and 1318 with control counts deducted after exposure to preservatives BIT, benzyl alcohol, phenoxyethanol. A) Variant counts and relative fold change in preservative susceptibility according to preservative type. B) Variant counts and relative fold change in preservative susceptibility according to strain type.

A t-test was conducted to compare variant abundance in experiments utilizing high and fast methodologies "H/F" (N = 11) and experiments utilizing low and slow methodologies "L/S" (N=9) experiments. There was a statistical difference in variant count observed in H/F methodologies (M= 23.73, SD= 18.93) and in L/S methodologies (M= 133.78, SD= 103.97); t(18)= 3.4614, p= 0.0028.

Weak preservative specific effects were observed in the average variant counts (minus controls) and preservative type. Across all strains and experimental conditions, variant counts for the preservative BIT (N=8, M=85, SD =119.55) were higher than for phenoxyethanol (N=6, M=78, SD=90.66) and benzyl alcohol (N=6, M=53, SD =31.61). However, there was no significant difference for variant count between preservatives as measured by a one-way anova; $F(2,17) = 0.224$, $p = 0.8014$.

Species specific effects on variant counts were not observed. Strain specific effects were observed in the average variant count (minus controls) across all preservatives and experimental conditions were seen for *B. cenocepacia* strain 1318. Variant counts for *B. cenocepacia* strain 1318 (N=4, M=35, SD =23.7) were lower than for *B. lat*a strain 1299 (N=4, M=83, SD=71.2), *B. cenocepacia* strain 1291 (N=8, M=81, SD =83.6) and *B. cenocepacia* strain 1292 (N=6, M=85, SD =134.8). Statistically significant differences for variant count between strains could not be measured by a one-way anova due to the small sample size.

5.4.3 Stable Preservative Resistance Variant Calling Analysis

There were variants in 75 genes with putative functions exclusively found in Bcc strains which demonstrated preservative induced stable adaptive resistance. Of these 75 genes containing variants, 13 genes with variants were found in more than one experiment and therefore had a higher likelihood of being selected rather than the product of random mutagenesis. Details for these 13 genes are given in Table 22.

5.4.3.1 The Effect of Preservative, Strain and Adaptive Evolution Method on Gene Variants Within Strains that Exhibited Stable Preservative Resistance

Of the 13 genes with variants, 10 were seen with L/S experiments, while 3 were shared with both L/S and H/F experiments. This is likely caused by the large number of mutations associated with L/S methodology.

In heatmap analysis, gene variants seen in more than one experimental condition did not cluster according to preservative or strain (Figure 32). With the exception of one gene, 12 of the genes with variants were seen across species and preservatives and could not be associated to a single condition. The only preservative specific gene variant associated with stable preservative resistance is *cqsA* (Table 22), a synthase of the autoinducer CAI-1 of the two-component quorum sensing (QS) system CAI-1/*CqsS* (Henke and Bassler, 2004). This gene variant is the only one involved in QS signalling seen in all preservative resistant or preservative exposed experiments. This gene variant is seen to be associated with BIT preservative only and indicates a preservative specific response. Further details on the function of this gene are given below.

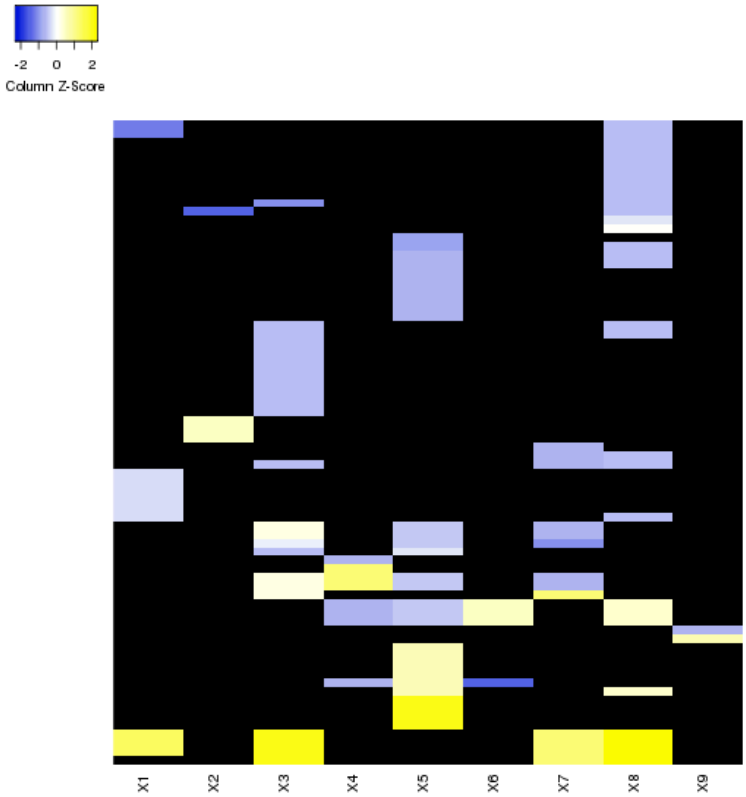


Figure 32: Heatmap of variant data (SNPs and Indels) for all 9 experiments which demonstrated stable preservative-induced adaptive resistance, less any variants also found in controls. Data for L/S benzyl alcohol 1299 and L/S phenoxyethanol 1318 are not displayed due to the large proportion of variants found. After filtering no variants were seen with H/F phenoxyethanol 1292 and H/F BIT 1292 and therefore these have not been included. Lanes 1-2 : H/F phenoxyethanol 1291 and 1292. Lanes 3-5: L/S phenoxyethanol 1291 and 1292, and 1299. Lane 6: H/F BIT 1291. Lanes 7-9: L/S BIT 1291, 1292 and 1318.

Table 22: Variants in genes observed in two or more experimental conditions which may contribute to stable adaptive resistance to preservatives

Gene	Protein	Description	Classification	Experimental Condition	Location	Effect	Effect Impact	Variant Type	UniprotKB Number
rsh	GTP pyrophosphokinase	Low Nutrient Signal	Stress Resistance	L/S BIT 1291 L/S PH 1299 L/S PH 1291	Cytoplasm	Downstream gene variant	MODIFIER	SNP INDEL	Q8YG65
rplK	50S ribosomal protein L11	Ribosome Stalk Protein	Stress Resistance / DNA Replication	L/S BIT 1292 L/S PH 1291	Cytoplasm	Upstream gene variant	MODIFIER	SNP	P0A7J7
srrA	Transcriptional regulatory protein	Low Oxygen Signal	Stress Resistance	L/S BIT 1292 L/S PH 1291	Cytoplasm	Upstream gene variant	MODIFIER	SNP	Q5HFT0
surE	5'-nucleotidase	Amino Acid Breakdown	Stress Resistance/ Metabolism	H/F PH 1318 L/S BIT 1292	Cytoplasm	Downstream gene variant	MODIFIER	SNP	Q9KI21
cqsA	CAI-1 autoinducer synthase	Quorum Sensing Autoinducer Synthesis	Signalling	L/S BIT 1291 L/S BIT 1292	Cytoplasm	Upstream gene variant	MODIFIER	SNP INDEL	A7N6R9
gpr	L-glyceraldehyde 3-phosphate reductase	Detoxification of methylglyoxal and L-glyceraldehyde 3-phosphate	Detoxification	L/S BIT 1292 L/S PH 1299	Cytoplasm	Upstream/ Downstream gene variant	MODIFIER	SNP INDEL	Q46851
iorA	Isoquinoline 1-oxidoreductase subunit alpha	Isoquinoline Degradation	Metabolism/ Detoxification	L/S BIT 1292 H/F PH 1291	Cytoplasm	Upstream/ Downstream gene variant	MODIFIER	SNP INDEL	Q51697
iorB	Isoquinoline 1-oxidoreductase subunit beta	Isoquinoline Degradation	Metabolism/ Detoxification	L/S BIT 1292 H/F PH 1291	Cytoplasm	Upstream/ Downstream gene variant	MODIFIER	SNP INDEL	Q51698
paaB	1 2 C2-phenylacetyl-CoA epoxidase 2C subunit B	Phenylacetate Degradation	Metabolism	L/S BIT 1291 L/S PH 1299 L/S PH 1292 L/S PH 1291	Cytoplasm	Upstream gene variant	MODIFIER	SNP	P76078

Table 22: Variants in genes observed in two or more experimental conditions which may contribute to stable adaptive resistance to preservatives (Continued)

Gene	Protein	Description	Classification	Experimental Condition	Location	Effect	Effect Impact	Variant Type	UniprotKB Number
paaC	1 2 C2-phenylacetyl-CoA epoxidase 2C subunit C	Phenylacetate Degradation	Metabolism	L/S BIT 1291 L/S PH 1299 L/S PH 1292 L/S PH 1291	Cytoplasm	Upstream gene variant	MODIFIER	SNP	P76079
paaD	4 2 C2-phenylacetyl-CoA epoxidase 2C subunit D	Phenylacetate Degradation	Metabolism	L/S BIT 1291 L/S PH 1299 L/S PH 1291	Cytoplasm	Upstream gene variant	MODIFIER	SNP	P76080
paaE	1 2C2-phenylacetyl-CoA epoxidase 2C subunit E	Phenylacetate Degradation	Metabolism	L/S BIT 1291 L/S PH 1299 L/S PH 1291	Cytoplasm	Upstream gene variant	MODIFIER	SNP	P76081
phnV	Putative 2-aminoethylphosphonate transport system permease protein	2-Aminoethylphosphonate Import	Metabolism	L/S BIT 1292 L/S PH 1299	Cell Wall	Upstream/ Downstream gene variant	MODIFIER	SNP INDEL	P96065

5.4.3.2 Putative Function of Gene Variants Associated with Stable Preservative Resistance

All 13 gene affecting variants are located in upstream or downstream genes and convey modifier effects which are unlikely to cause a change in core functions. Gene grouping is difficult as some of these genes demonstrate cross functionality, for example, various genes exhibit a secondary function under stress conditions. The 13 genes can be loosely clustered into 4 functional groups:

1. Stress resistance (4)
2. Signaling (1)
3. Detoxification (1)
4. Metabolism (7)

Stress Resistance

Two genes are associated with the synthesis of the alarmone guanosine tetra/penta phosphate (p)ppGpp: *rsh* and *rplk*. *Rsh* is a GTP (guanosine tetra/penta phosphate) pyrophosphokinase (Dozot *et al*., 2006). *rplK* is a 50S ribosomal protein called L11 which mediates the activity of a *relA* a GTP (guanosine tetra/penta phosphate) pyrophosphokinase (Jenvert and Schiavone, 2007; Yang and Ishiguro, 2001).

srrA is a transcriptional regulatory protein of the two-component regulatory system *SrrA/SrrB* in *Staphylococcus aureus* (Pragman *et al*., 2004). Both *srrA/srrB* and the homologue *ResD/ResE* in *Bacillus subtilis* are activated under low oxygen availability (hypoxic stress) and are involved in nitric oxide stress resistance (Fang *et al*., 2016).

SurE is a 5'-nucleotidase belonging to the protein superfamily of *surE* phosphatase/nucleotidase superfamily (Proudfoot *et al*., 2004). These enzymes are involved in the hydrolysis of nucleotide phosphate groups, which is important for amino acid metabolism. In *Bacillus subtilis* and *Escherichia coli* the activity of *surE*-like 5′-nucleotidases are involved in acid and oxidative stress responses (Terakawa *et al*., 2016).

Signalling

cqsA is a synthase of the quorum-sensing autoinducer CAI-1 which is sensed by *cqsS* (Lorenz *et al*., 2017). The CAI-1/*CqsS* QS system works in parallel with other quorum sensing (QS) systems but is induced particularly at low cell densities. In *V. cholerae* CAI-1/*CqsS* QS regulates of a collection of genes for bioluminescence, a type III secretion system, and metabolically significant proteins such as metalloprotease (Henke and Bassler, 2004).

Degradation of Toxins

Gpr is a L-glyceraldehyde 3-phosphate reductase (Kalyananda *et al*., 1987). Its primary function is in detoxification of L-glyceraldehyde 3-phosphate (L-GAP) and methylglyoxal which are seen to decrease cell viability at high intracellular levels (Desai and Miller, 2008; Ko *et al*., 2005; Grant *et al*., 2003). L-GAP and methylglyoxal are by products of carbohydrate metabolism. Methylglyoxal is also formed from the oxidation of DNA and RNA by strong acids (Chaplen *et al*., 1996).

Metabolism

iorA/iorB form an oxidoreductase complex capable of the breakdown of aromatic organic compounds such as isoquinoline, isoquinolin-5-ol, phthalazine and quinazoline (Lehmann *et al*., 1995).

paaABCDE complex is a well-characterised cluster of *paa* (phenylacetate acid) degradation enzymes which enable bacterial phenylacetate degradation(Fernández *et al*., 2006). These genes are also seen to play an essential role in Bcc stress with host colonisation (Yudistira *et al*., 2011; Hamlin *et al*., 2009). The phenylacetate degradation pathway has also been associated with *B. cenocepacia* adaptive resistance to the antibiotic meropenem (Bazzini *et al*., 2011).

phnV is a putative 2-aminoethylphosphonate transport system permease protein and a component of the multi-subunit ABC transporter *PhnSTUV* (InterPro, 2019). 2-aminoethylphosphonate (ciliatine) is a common phosphonate compound found in the environment and once imported into the cell can be metabolised and used as a carbon-phosphorous source for various cellular processes including growth and energy (McClelland *et al*., 2004).

Summary

There were 13 genes associated with preservative induced stable adaptive resistance which were seen in more than one experiment. Of the 13 genes, none were strain specific and only one gene (*cqsA*) was specific to the preservative BIT. Variants affecting *cqsA* are most likely a BIT induced adaptive resistance mechanism to initiate QS signalling of bacterial survival and virulence factors when exposed to BIT preservatives.

10 genes with variants were seen with L/S experiments only, while 3 were shared with both L/S and H/F experiments. This is likely caused by the larger number of gene mutations associated with L/S methodology.

The 13 genes were loosely clustered into 4 functional groups. Of the 13 genes with variants, the majority (7/13) were concerned with metabolism. Gene variants are seen in phenylacetate degradation pathway (*paaBCDE*) and carbon metabolism pathways (*phnV*), both are known to be involved in antimicrobial resistance. Variants are also seen in aromatic compound degradation (*iorA/iorB*) which indicates the involvement of enzymatic breakdown in preservative adaptive resistance.

Of the 13 genes with variants, four were concerned with stress resistance. Two genes are involved in alarmone synthesis (*rsh* and *rplk*) which in the context of stable adaptive resistance to biocides, may be acting to induce bacterial dormancy and to signal cellular changes in response to toxic preservatives. Two genes (*srrA* and *surE*) are known to be involved in oxidation stress which is likely to be an adaptive stress response to enhance cellular survival when exposed to oxidative preservative agents.

Gpr is an oxidoreductase of the aldo-keto reductase (AKR) enzymes seen to be involved in the detoxification of toxic substances generated from natural carbon metabolism and nucleic acid oxidation. It is likely involved in the detoxification of toxic intercellular metabolites caused by preservative mediated oxidative DNA damage and cellular induced carbohydrate metabolism.

5.4.4 Gene Variants Within Strains Exposed to Preservative

In total, experimental data was available for 20 samples. There were 157 gene hitting variants shared across 2 or more experimental conditions not found in controls. Of these there were 83 genes with unknown functions and 74 genes with names and putative functions. After filtering no variants were seen with H/F phenoxyethanol 1292 and H/F BIT 1292 and therefore these have not been included. Data for L/S benzyl alcohol 1299 and L/S phenoxyethanol 1318 are not displayed due to the large proportion of variants found. Of these 74 genes, 43 genes had normal cellular functions not known or presumed to be involved with resistance. Details on the 43 genes involved in normal cellular functions can be found in appendix 3. These genes have been disregarded and the subsequent research surrounds the 31 genes with cellular functions previously identified as involved in antimicrobial resistance.

5.4.4.1 The Effect of Preservative, Strain and Evolution Method on Gene Variation Within Strains Exposed to Preservative

A heatmap of the 20 experimental samples and 74 genes demonstrated pronounced clustering of L/S experiments but little clustering of H/F experiments (Figure 33). Of the 31 gene variants seen in preservative exposure, 17 gene variants were shared with H/F and L/S methodologies, 12 were seen in L/S experiments only and 2 were seen in H/F experiments only. Of the 17 genes shared across L/S and H/F experiments the majority of these involved genes concerning efflux pumps (N=5) and damage responses (N=4). Incidentally, genes concerning efflux and damage responses are not seen exclusively in L/S or H/F. This demonstrates the importance of these genes in general mechanisms of bacterial adaptive resistance to preservatives. Of the 12 genes seen in L/S experiments only, the majority (7/12) concern stress resistance. This illustrates the importance of stress resistance to survival when grown in sub-inhibitory concentrations of preservatives for long periods. The two genes seen in H/F experiments only are both seen to contribute to the degradation of toxic substances. This indicates that toxic substance catabolism is an important mechanism to cellular survival during short exposure to high concentrations of preservatives.

Of the 31 gene variants only 6 clustered by preservative type, with four associated with BIT and two associated with benzyl alcohol. The four genes associated with

BIT preservative were also *B. lata* 1299 strain type selective. Phenotypically *B. lata* 1299 strain exhibited increased BIT resistance when compared to the 47 *Burkholderia* strain panel (See section 4.4.1 Preservative susceptibility of a panel of *Burkholderia* strains to nature identical and synthetic preservatives systems). Three of the four genes associated with BIT preservative were involved in damage response : *ada/alkA* involved in alkylation damage (Table 27) and *speG* involved in the degradation of toxic polyamines (Table 24). The remaining gene, *lutP* is involved in biofilm synthesis (Table 28).
The two genes associated with benzyl alcohol preservative were both associated with efflux: *ttgR* involved in solvent efflux transcription and *mexA* a multidrug efflux pump membrane fusion protein (MFP). Interestingly, another efflux pump component *acrB* is also seen with four benzyl alcohol preservative experiments and one phenoxyethanol experiment. Further details on the functions of these genes are given in section 5.4.4.2. For benzyl alcohol preservative experiments both *mexA* and *acrB* acquire a function affecting mutation. Efflux pumps are considered to be significant for bacterial adaption to antimicrobials. Reduction in susceptibility to benzyl alcohol preservative and *B. cenocepacia* strains could not be induced in all experiments and was only transiently increased in *B. lata* strain 1299 L/S experiments. It could be that benzyl alcohol induces mutation of efflux pump constituents which increases preservative efficacy.

Due to the lack of multiple strains of *B. lata*, species selective gene variants could not be asserted. In total 6 genes clustered by *B. lata* 1299 strain type (Figure 33). Four of these were also BIT selective as discussed previously. The remaining two genes containing variants were *srpC* a solvent efflux pump outer membrane protein (Table 25) and *soxR* a redox-sensitive transcriptional activator triggered by superoxide (Table 26). The function of these 6 genes with variants were varied and were involved in different cellular functions. *B. cenocepacia* species level selectivity for gene variants was not observed. Unique gene variants were associated with *B. cenocepacia* strain types 1291 (N=3) and 1318 (N=2). No unique gene variants were associated with *B. cenocepacia* strain 1292. For *B. cenocepacia* strain 1291 the three strain unique genes were *hrp1* a hypoxic response protein (Table 23), *hxlR* a transcriptional activator for a biochemical pathway involved in toxic compound degradation (Table 24) and *puuA* a gamma-glutamylputrescine synthetase involved

in putrescine degradation pathway (Table 24). For *B. cenocepacia* strain 1318 the two strain unique genes were *sadA* a trimeric autotransporter adhesin located in the outer membrane which enables adhesion (Table 28) and *dnaE* an error-prone DNA polymerase (see Table 23). Further details on the functions of these genes are given in section 5.4.4.2. The function of these *B. cenocepacia* strain specific genes with variants were varied and involved in different cellular functions.

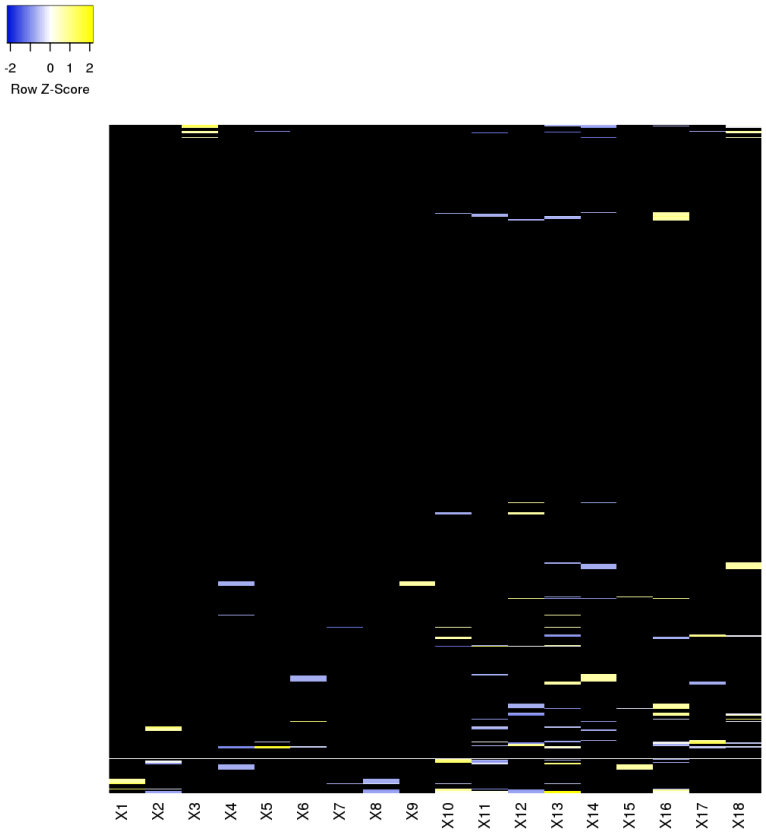


Figure 33: Heatmap of variant data (SNPs and Indels) for all 18 experimental samples less any variants also found in controls. Lanes 1-4 : H/F benzyl alcohol 1291, 1292, 1299 and 1318. Lanes 5-7: H/F BIT 1291, 1299 and 1318. Lanes 8-9 : H/F phenoxyethanol 1291 and 1318. Lanes 10-11 : L/S benzyl alcohol 1291 and 1292. Lanes 12-15 : L/S BIT 1291, 1292, 1299 and 1318. Lanes 16-18 : L/S phenoxyethanol 1291 and 1292, and 1299.

5.4.4.2 Putative Function of Gene Variants of Preservative Exposed Bcc Strains

Categorically assigning genes to a functional group is difficult due to the utility of proteins in various processes. However, the 31 genes containing variants can be loosely clustered into 6 functional groups:

1. Stress resistance (9)
2. Degradation of toxins (7)
3. Efflux pump systems (5)
4. Cellular membrane (2)
5. Damage response (4)
6. Biofilm (4)

Of the 31 genes with variants, 25 gene affecting variants are located in upstream or downstream genes and convey modifier effects which are unlikely to cause a change in core functions. The variants in 6 genes that had moderate or high impacts were seen across 7 different experimental conditions (Table 23). These higher impact variants could cause a change to the protein and its function possibly rendering it dysfunctional. Moderate and high impact variants are seen most commonly with L/S experiments, presumably due to the higher number of variants associated with this method. The majority (5/7) of these moderate or high impact variants were associated with benzyl alcohol preservative which did not display a change in preservative susceptibility. Of the 5 genes associated with benzyl alcohol and moderate/high impacts, 4 of the genes are cell membrane components.
Although, the function of variants cannot be fully understood without further experimentation, it is likely that *soxR* does not play a role in bacterial adaptive resistance due to the presence of moderate and high impact variants in both experimental conditions.

Table 23: **Genes containing variants which cause a "High" or "Moderate" impact on function and the adaptive resistance condition within which they were observed.**

Gene	Classification	Condition	Variant	Impact
mexA	Efflux pump systems	H/F BA 1292	Stop gained	HIGH
soxR	Damage response	H/F BA 1299	Missense variant	MODERATE
soxR	Damage response	L/S PH 1299	Missense variant Stop gained	MODERATE HIGH
kdsA	Cellular membrane	L/S BA 1291	Conservative in-frame insertion	MODERATE
ompR	Stress resistance	L/S BA 1292	Missense variant	MODERATE
acrB	Efflux pump systems	L/S BA 1291	Missense variant	MODERATE
dnaE	Stress resistance	L/S BIT 1318	Missense variant Frameshift variant	MODERATE HIGH

Abbreviations:
BA, Benzyl Alcohol
PH, Phenoxyethanol
BIT, Benzisothiazolinone
1291, *B. cenocepacia* strain 1291
1292, *B. cenocepacia* strain 1292
1318, *B. cenocepacia* strain 1318
1299, *B. lata* strain 1299
H/F, High and Fast
L/S, Low and Slow

Stress Resistance

Of the 31 genes containing variants the majority are functionally involved in cellular stress resistance responses (9/31). Further details of the genes with variants are given in Table 24.

The *envZ/ompR* signalling pathway consists of *envZ* a histidine kinase sensor with domains in the periplasm and cytoplasm, and *ompR* a transcriptional regulatory protein (Delgado *et al*., 1993). Osmolarity changes in the cytoplasm activate phosphorylation of *envZ*. *EnvZ* is able to control phosphatase activity based on osmolarity, which triggers the differential expression of the outer membrane porins *ompF* and *ompC*. This highly regulated transcription system is seen to respond to various external changes other than osmolarity such as pH, temperature and nutritional stress (Jaworska *et al*., 2018). The transcriptional factor *ompR* has also been implicated in other cellular processes in pathogenic bacterial species such as the expression of virulence genes in *Shigella flexneri* (Bernardini *et al*., 1990) and *Salmonella typhimurium* (Chatfield *et al*., 1991), expression of a secretion controlling two-component signalling pathway in *Salmonella* spp. (Feng *et al*., 2003), acid tolerance in *Salmonella enterica* (Bang *et al*., 2002) and *Escherichia coli* (Stincone *et al*., 2011), flagellum expression in *Escherichia* (Shin and Park, 1995), and antibiotic resistance in *Salmonella typhimurium* (Knopp and Andersson, 2018) and *Escherichia coli* (Hirakawa *et al*., 2003).

Hrp1 is a hypoxic response protein from the model organism *Mycobacterium tuberculosis* (Sun *et al*., 2017). Environmental stress conditions such as reduced or poor oxygen levels, high nitric oxide and carbon monoxide triggers a 48 gene regulation response activated by *DosR/DosS* two-component regulatory system (Sivaramakrishnan and de Montellano, 2013). This response is seen to functionally attenuate bacterial replication and induce a dormant (viable but not culturable state) which attributes to long term survival (Voskuil *et al*., 2003). Of the 48 genes regulated by *DosR/DosS*, transcription of hypoxic response protein is strongly expressed (Sherman *et al*., 2001).

The *hslO* gene codes for a 33 kDa chaperonin (HSP33) which is a molecular chaperone belonging to large family of stress reduction proteins called Heat Shock

Proteins (Jakob *et al*., 1999). HSP33 is activated under oxidative stress and heat shock conditions common when cells are treated with aggressive antioxidants such as biocides (Cremers *et al*., 2010). Under these fast-acting antioxidants, cellular ATP and ATP dependent molecular chaperone activity drops, however HSP33 is ATP independent, and acts as a bacterial stress defence, protecting against stress-induced protein unfolding and aggregation (Jakob *et al*., 1999).

htrA otherwise known as *degP* encodes a member of the serine protease enzymes family. The *htrA/degP* encoded enzymes are (arguably) bifunctional, displaying a primary function as proteolysis enzymes at various temperatures and display a secondary weaker function as low temperature dependent molecular chaperones which mediate the folding/re-folding of proteins (Chang, 2016). The breakdown of irreversibly denatured and misfolded proteins is essential to prevent toxic protein aggregation in the cytoplasm and therefore cell survival in stress conditions (Isaac *et al*., 2005). *htrA* (high temperature requirement) gene was first identified as a thermoprotective response but has also been implicated in other stress responses such as changes in pH, osmotic gradients, acid tolerance and oxidation resistance (Sebert *et al*., 2005).

The *clcA* gene encodes for the H(+)/Cl(-) exchange transporter protein (Iyer *et al*., 2002). This transporter is of the CLC family of chloride transporters and works as an antiporter system to co-ordinate the intake of two chloride molecules Cl(-) with the expulsion of a hydrogen proton H(+) (Jayaram *et al*., 2008). There are molecular "gates" placed at both ends of this channel, and the inner gate is resistant to pH changes and enables cellular acid tolerance.

cmpR is an HTH-type transcriptional activator in the cyanobacteria *Synechococcus* strain PCC 7942 (Mahounga *et al*., 2018). *cmpR* controls a four component ABC transporter system called *cmpABCD* which controls bicarbonate transportation under poor environmental carbon dioxide availability. Under reduced carbon dioxide conditions, the *cmpR* activator binds to the regulatory region of *cmpA, activating transcription of* the *cmpABCD* operon (Omata *et al*., 2001).

ComEC is an operon protein of the competence related DNA transformation transporter protein family. In the model organism *Bacillus subtilis*, *comEC* is required for the incorporation of foreign DNA of late-competence stage cells by facilitating DNA binding to the cell surface and transporting through the membrane (Hahn *et al*., 1993).

dnaE2 is an error-prone DNA polymerase which is able to replicate from lesion damaged DNA in a process called translesion synthesis (TLS) (Seitz and Blokesch, 2014). It is thought that bacterial populations under stress conditions trigger the upregulation of error prone DNA polymerases as part of a SOS response, to allow damage tolerance and increase the mutation rate to allow population persistence through genetic adaption (Tegova *et al*., 2004). This is well characterised in *E .coli* whereby expression of error-prone polymerases Pol V and Pol IV are induced during SOS response (Bruck *et al*., 2003). The inhibition of the SOS response in *E. coli* results in the reduction of mutations and increased sensitivity to antibiotics (Cirz *et al*., 2005).

Table 24: SNP/INDEL information for genes in two or more experimental conditions involved in stress resistance

Gene	Protein	Description	Location	Experimental Conditions	Effect	Effect Impact	Variant Type	UniprotKB Number
clcA	H(+)/Cl(-) exchange transporter	Chloride Transport	Cell Wall	L/S BIT 1299 L/S PH 1292	Downstream gene variant	MODIFIER	SNP	Q8ZRP8
cmpR	HTH-type transcriptional activator	Bicarbonate Transportation	Cell Wall	L/S BIT 1299 L/S PH 1292 H/F BIT 1299	Upstream gene variant	MODIFIER	SNP/INDEL	Q55459
comEC	ComEC operon protein 3	Uptake of Transforming DNA	Cell Wall	L/S BA 1291 L/S BIT 1292	Downstream gene variant	MODIFIER	SNP/INDEL	P39695
dnaE	Error-prone DNA polymerase	DNA Polymerase	Cytoplasm	H/F BA 1318 L/S BIT 1318	Upstream/Downstream gene variant Synonymous variant Missense variant Frameshift variant	MODIFIER LOW MODERATE HIGH	SNP/INDEL	B8H427
envZ	Osmolarity sensor protein	Osmoregulator	Cell Wall	L/S BA 1292 L/S BIT 1299	Upstream gene variant	MODIFIER	SNP	P0AEJ4
hrp1	Hypoxic response protein 1	Cellular Replication Altering Stress Response	Cytoplasm	L/S BA 1291 L/S PH 1291	Upstream /Downstream gene variant	MODIFIER	SNP/INDEL	P9WJA2
hslO	33 kDa chaperonin	Heat Shock Protein	Cytoplasm	L/S BA 1291 L/S BIT 1292	Downstream gene variant	MODIFIER	SNP/INDEL	P0A6Y5
htrA	Putative serine protease	Proteolysis Enzymes	Cytoplasm	L/S BA 1292 L/S BA 1291 L/S BIT 1292	Downstream gene variant	MODIFIER	SNP/INDEL	P73354
ompR	Transcriptional regulatory protein	Osmoregulator	Cytoplasm	L/S BA 1292 L/S BIT 1299	Missense variant	MODERATE	SNP	P0AA16

Degradation of Toxins

Of the 31 genes containing variants seven are functionally involved in the degradation of toxins. Further details of the genes with variants are given in Table 25.

acoD is an acetaldehyde dehydrogenase involved in acetonin and ethanol degradation in *Cupriavidus metallidurans* (Priefert *et al*., 1992).

apc3 is a subunit of an acetophenone carboxylase, which forms benzoylacetate from acetophenone in the degradation of ethylbenzene (Jobst *et al*., 2010).

benM is an HTH-type transcriptional regulator involved in benzoate degradation (Ezezika *et al*., 2007).

hxlR is an HTH-type transcriptional activator for the ribulose monophosphate (RuMP) pathway for metabolism of toxic compounds and compound intermediates such as formaldehyde, methane and methanol (Yurimoto *et al*., 2005). *Burkholderia cepacia* TM1 is also seen to contain *hxlR* homologues however further investigation on the actual regulatory function of *hxlR* has not been executed (Mitsui *et al*., 2003).

speG is a spermidine N(1)-acetyltransferase which catalyses the transfer of acetyl groups from acetyl co-enzyme A to the amino groups of spermidine and other toxic polyamines in the model organism *E. coli strain* K12 (Limsuwun and Jones, 2000; Matsui *et al*., 1982). This acetylation process removes the positive charge of the polyamines, aillowing it to be expelled by the cell. Aggregation of cellular polyamines results in reduced protein synthesis and decreased cell viability (Fukuchi *et al*., 1995).

puuA is a gamma-glutamylputrescine synthetase and *puuC* is a NADP/NAD-dependent aldehyde dehydrogenase both used in the four-step putrescine degradation pathway whereby putrescine/ spermidine is broken down into nitrogen and carbon for growth (Kurihara *et al*., 2005).

Table 25: **SNP/INDEL information for genes in two or more experimental conditions involved in the degradation of toxins**

Gene	Protein	Description	Location	Experimental Condition	Effect	Effect Impact	Variant Type	UniprotKB Number
acoD	Acetaldehyde dehydrogenase 2	Ethanol Metabolism	Cytoplasm	H/F BA 1318 H/F PH 1318 L/S BA 1292	Upstream gene variant	MODIFIER	INDEL	P46368
apc3	Acetophenone carboxylase gamma subunit	Ethylbenzene Metabolism	Cytoplasm	L/S BA 1291 L/S BIT 1292 L/S BIT 1291 L/S PH 1291 H/F BA 1292 H/F PH 1291	Downstream gene variant	MODIFIER	INDEL	Q5P5G4
benM	HTH-type transcriptional regulator	Benzoate Degradation	Cytoplasm	L/S BA 1291 L/S BIT 1299	Upstream/Downstream gene variant	MODIFIER	INDEL	O68014
hxlR	HTH-type transcriptional activator	Formaldehyde Degradation	Cytoplasm	H/F BA 1291 H/F PH 1291	Upstream gene variant	MODIFIER	SNP	P42406
puuA	Gamma-glutamylputrescine synthetase	Polyamine Degradation	Cytoplasm	L/S BA 1291 L/S PH 1291	Upstream gene variant	MODIFIER	SNP/INDEL	P78061
puuC	Aldehyde dehydrogenase	Polyamine Degradation	Cytoplasm	L/S BA 1292 L/S PH 1299 L/S PH 1291	Upstream gene variant	MODIFIER	INDEL	P23883
speG	Spermidine N(1)-acetyltransferase	Polyamine Degradation	Cytoplasm	L/S BIT 1299 H/F BIT 1299	Downstream gene variant	MODIFIER	INDEL	P0A951

Efflux Pumps

Of the 31 genes containing variants five are functionally involved in efflux pumps. Further details of the genes with variants are given in Table 26.

acrB is a multidrug efflux pump transporter of the tripartite efflux pump *AcrA/AcrB/TolC* (Hobbs *et al*., 2012). In *E. coli* strain K12 the outer membrane factor *TolC* is associated with the inner membrane protein from the RND family called *acrB*, and the membrane fusion protein *acrA* (Sennhauser *et al*., 2006).

bepE is an efflux pump membrane transporter that forms part of a tripartite efflux pump *bepCDE* (Li and Nikaido, 2009). In *Brucella suis* the tripartite efflux pump *bep* (Brucella efflux protein) exists, with an outer membrane factor called *bepC*, an inner membrane protein from the resistance-nodulation-cell division (RND) family called *bepE* and a membrane fusion protein *bepD* (Martin *et al*., 2009).

mexA is part of the *mexA/mexB/oprM* complex, a major component of multidrug resistance enabling efflux systems in *Pseudomonas* (Yoneyama *et al*., 2000). *MexB* is the inner membrane protein, which is connected to *MexA* the membrane fusion protein, which is connected to the outer membrane factor *oprM* .

srpC is a solvent efflux pump outer membrane protein in the efflux pump *SrpABC* in the organism *Pseudomonas putida strain S12* (Kieboom *et al*., 1998). The outer membrane component *srpC* is connected to the membrane fusion protein *srpA* and the inner membrane protein *srpB*. This pump is homologous to the *ttgGHI* toluene efflux pumps of *Pseudomonas putida DOT-T1E* (Ramos *et al*., 2015).

ttgR is a transcriptional repressor which controls expression of *ttgABC* toluene efflux complex in *Pseudomonas putida DOT-T1E* (Rojas *et al*., 2001) . In the presence of chloramphenicol and tetracycline antibiotics, the *ttgR* protein disassociates from the promoter region of *ttgABC* to induce expression (Terán *et al*., 2003).

Table 26: SNP/INDEL information for genes in two or more experimental conditions involved in efflux pumps

Gene	Protein	Description	Location	Experimental Condition	Effect	Effect Impact	Variant Type	UniprotKB Number
acrB	Multidrug efflux pump subunit	Antimicrobial Efflux Pump	Cell Wall	H/F BA 1291 H/F BA 1292 L/S BA 1291 L/S BA 1292 L/S PH 1291	Upstream/ Downstream gene variant / Missense variant	MODIFIER MODERATE	SNP/INDEL	P31224
bepE	Efflux pump membrane transporter	Antimicrobial Efflux Pump	Cell Wall	H/F BA 1299 L/S BIT 1292 L/S PH 1299	Upstream gene variant	MODIFIER	SNP	Q8G2M6
mexA	Multidrug resistance protein	Antimicrobial Efflux Pump	Cell Wall	H/F BA 1292 L/S BA 1291 L/S BA 1292	Upstream/ Downstream gene variant / Stop gained	MODIFIER HIGH	SNP/INDEL	P52477
srpC	Solvent efflux pump outer membrane protein	Toxic Efflux Pump	Cell Wall	H/F BA 1299 L/S PH 1299	Upstream gene variant	MODIFIER	SNP	O31101
ttgR	HTH-type transcriptional regulator	Efflux Pump Regulator	Cell Wall	H/F BA 1292 L/S BA 1291 L/S BA 1292	Upstream gene variant	MODIFIER	SNP/INDEL	Q9AIU0

Cell Membrane

Of the 31 genes containing variants two are functionally involved in cellular membrane activity. Further details of the genes with variants are given in Table 27.

kdsA is a 2-dehydro-3-deoxyphosphooctonate aldolase involved in 3-deoxy-D-manno-octulosonate biosynthesis, which is a part of the lipopolysaccharide biosynthesis pathway, essential for outer membrane biogenesis (Goldman and vine, 1987). UniProtKB has automatically assigned this to strains of *Burkholderia cenocepacia* (strain ATCC BAA-245 / DSM 16553 / LMG 16656 / NCTC 13227 / J2315 / CF5610) and *Burkholderia cepacia* (J2315) based on its sequence homology to other species with 2-dehydro-3-deoxyphosphooctonate aldolase.

SadH is a putative oxidoreductase, and whilst no studies exist on its action, functional similarity can be inferred from the *mymA* homologue in the same model organism, *Mycobacterium tuberculosis* (Singh *et al*., 2003). *mymA* is seen to have a significant effect of the architecture of the cell envelope through changing the composition of the long chain fatty acids (mycolic acids) that are a key constituent of the structure (Singh *et al*., 2005).

Table 27: SNP/INDEL information for genes in two or more experimental conditions involved in the cell membrane structure

Gene	Protein	Description	Location	Experimental Condition	Effect	Effect Impact	Variant Type	UniprotKB Number
kdsA	2-dehydro-3-deoxyphosphooctonate aldolase	Outer Membrane Biogenesis	Cytoplasm	L/S BA 1291 L/S BIT 1292 L/S PH 1299 H/F BA 1318	Upstream gene variant / Conservative in-frame insertion	MODIFIER MODERATE	SNP/INDEL	B4EDA2
sadH	Putative oxidoreductase	Cell Envelope Influencer	Cell Wall	H/F BIT 1291 H/F BIT 1299 L/S BIT 1292 L/S PH 1292 L/S PH 1299	Downstream gene variant	MODIFIER	SNP	P9WGP8

Damage Response

Of the 31 genes containing variants four are functionally involved in cellular damage responses. Further details of the genes with variants are given in Table 28.

Ada and *alkA* are genes involved in the bacterial response to alkylation damage, called the *Ada* response. Alkylation causes the addition of an alkyl group, this often causes lesions in DNA molecules and give rise to cell mutagenesis and cytotoxic effects (McCarthy and Lindahl, 1985). *Ada* is a dual functional transcriptional activator and DNA repair enzyme. *AlkA* is a DNA-3-methyladenine glycosylase II which repairs and removed wide range of alkylated bases. (Mielecki and Grzesiuk, 2014).

RclR is a reactive chlorine species (RCS) specific HTH-type transcriptional activator in *E. coli*. Reactive chlorine species are highly reactive chlorinating oxidants and can cause significant cytotoxic effects. *RclR* expression is activated through oxidation by RSC, this triggers further *rclR* transcriptional activation and activation of RSC resistance genes *rclA*, *rclB*, and *rclC* (Parker *et al*., 2013).

soxR is a redox-sensitive transcriptional activator triggered by superoxides and nitric oxide. Superoxides (reactive anion species) and nitric oxide (free radical) are types of reactive oxygen species (ROS) formed as natural by-products of cellular processes which cause non-specific damage to cellular DNA and proteins (Koo *et al*., 2003).

Table 28: SNP/INDEL information for genes in two or more experimental conditions involved in damage response

Gene	Protein	Description	Location	Experimental Condition	Effect	Effect Impact	Variant Type	UniprotKB Number
ada	Bifunctional transcriptional activator/DNA repair enzyme	DNA Repair Activation and Enzyme	Cytoplasm	H/F BIT 1299 L/S BIT 1299	Downstream gene variant	MODIFIER	INDEL	P06134
alkA	DNA-3-methyladenine glycosylase 2	DNA Repair Enzyme	Cytoplasm	H/F BIT 1299 L/S BIT 1299	Downstream gene variant	MODIFIER	INDEL	P04395
rclR	RCS-specific HTH-type transcriptional activator	Reactive Chloride Species Response	Cytoplasm	H/F BA 1291 H/F BA 1292 L/S BA 1291 L/S BA 1292 L/S BIT 1291 L/S BIT 1292 L/S PH 1291	Downstream gene variant	MODIFIER	SNP	P77379
soxR	Redox-sensitive transcriptional activator	Superoxide Sensor	Cytoplasm	H/F BA 1299 L/S PH 1299	Missense variant/ Stop Gained	MODERATE HIGH	SNP	P0ACS2

Biofilm

Of the 31 genes containing variants four are functionally involved in biofilm activity. Further details of the genes with variants are given in Table 29.

Gmr (*PdeR*) codes for a cyclic di-GMP phosphodiesterase, which is a secondary nucleotide signalling messenger which forms a part of a signalling cascade which ultimately controls the expression of *csgD* a biofilm regulator and key component to the production of amyloid curli fibres (Lindenberg *et al*., 2013). Cyclic di-GMP is mediated by diguanylate cyclases (DGC) and degraded by specific phosphodiesterases (PDEs). In the model organism *E. coli* strain *K12*, cyclic di-GMP is controlled by two production/degradation paired modules, DgcE/PdeH pair (module I) which in-turn regulates *DgcM/PdeR* pair (module II), which stimulates the activity of transcription factor *MlrA* which then regulates *csgD expression* (Pratt *et al*., 2009).

lutP is a L-lactate permease which is a transport membrane protein that facilitates the uptake of L-lactate for metabolism by *lacABC* operon encoding L-lactate metabolism genes (Chai *et al*., 2009). L-lactate is used as a source for carbon for growth in various bacteria and is also required for biofilm formation in the model organism *Bacillus subtilis*. In *Bacillus subtilis* control of *lutP* is regulated by two repressors, the upstream *lutR* gene and also *SinR* repressor, the principal mechanism of biofilm formation during growth (Chiu *et al*., 2014).

mcbR is an HTH-type transcriptional regulator which increases biofilm formation in the model organism *E. coli* strain *K12* (Zhang *et al*., 2008). In this complex system, expression of *mcbR* is triggered by a quorum sensing signal autoinducer (AI-2) which stimulates expression of *mqsR* a flagellum motility activator, which in-turn activates the HTH-type transcriptional regulator *mcbR*. Expression of *mcbR* represses transcription of *mcbA* a periplasmic protein which increases exopolysaccharide colonic acid and mucoidy therefore increasing biofilm formation (García-Contreras *et al*., 2008).

sadA is a trimeric autotransporter adhesin. Trimeric autotransporter adhesins are outer membrane proteins which facilitate adhesion to surfaces. Trimeric

autotransporter adhesins are found in many pathogenic gram-negative bacterial species and are paramount for host colonisation and infection (Bassler *et al*., 2015). *sadA* expression in the model organism *Salmonella enterica* is seen to promote cellular aggregation and biofilm synthesis (Raghunathan *et al*., 2011). This is also mirrored by a trimeric autotransporter adhesin *upaG* in *E. coli,* which both facilitated in vitro binding to bladder epithelial cells, but also caused cellular aggregation and biofilm synthesis (Valle *et al*., 2008).

Table 29: SNP/INDEL information for genes in two or more experimental conditions involved in biofilms.

Gene	Protein	Location	Effect	Experimental Condition	Effect Impact	Variant Type	UniprotKB Number
gmr	Cyclic di-GMP phosphodiesterase	Cytoplasm	Downstream gene variant	H/F BA 1292 H/F PH 1291 L/S BA 1291 L/S BA 1292 L/S BIT 1291 L/S BIT 1292 L/S PH 1291	MODIFIER	INDEL	P77334
lutP	L-lactate permease	Cell Wall	Upstream gene variant	H/F BIT 1299 L/S BIT 1299	MODIFIER	INDEL	P71067
mcbR	HTH-type transcriptional regulator	Cytoplasm	Downstream gene variant	L/S BIT 1299 L/S PH 1291	MODIFIER	SNP	P76114
sadA	Autotransporter adhesin	Cell Wall	Downstream gene variant	H/F BA 1318 L/S BIT 1318	MODIFIER	SNP/INDEL	Q8ZL64

Summary

In total, experimental data was available for 20 samples. There were 74 genes with names and putative functions shared across 2 or more experimental conditions and not found in controls. Of these there were 31 genes with cellular functions previously identified as involved in antimicrobial resistance.

Adaptive resistance method effects were seen to correlate weakly with gene functions. Of the 31 gene variants known to be involved in resistance, 17 gene variants were shared with H/F and L/S methodologies. The majority of these involved genes concerning efflux pumps (N=5) and damage responses (N=4) and indicate these cellular processes are essential for broad bacterial adaptive resistance to preservatives. Of the 12 genes seen in L/S experiments only, the majority (7/12) concern stress resistance which illustrates the importance of stress resistance to survival when grown in sub-inhibitory concentrations of preservatives for long periods. The two genes seen selectively in H/F experiments are both seen to contribute to the degradation of toxic substances which indicates toxic substance catabolism is an important mechanism for cellular survival during short exposure to high concentrations of preservatives.

Out of 31 genes only 6 were associated with specific preservatives. Preservative specific association could not be attributed to phenoxyethanol preservatives. The four genes associated with BIT were also associated with the highly BIT resistant *B. lata* strain 1299. These genes were diverse in function and concerned biofilm synthesis, alkylation damage and degradation of toxic polyamines.
Two genes were only associated with benzyl alcohol preservative and were both associated with efflux pumps. Efflux is considered a driving mechanism of antimicrobial resistance to benzyl alcohol preservatives. Decreased susceptibility to benzyl alcohol could not be induced. It could be that benzyl alcohol selects efflux pump mutations, which increases its biocidal effect. This is also supported by the observation that of the 7 experimental conditions which have a moderate/high impact effect, benzyl alcohol preservative treatment accounts for five of them (Table 23).

Species specific gene variants were not observed. There were strain specific gene variants for *B. cenocepacia* strain 1291 (N=3) and 1318 (N=2) , and *B. lata* strain

1299 (N=6) however for *B. cenocepacia* strain 1292 selective gene variants were not observed. The strain specific genes all had very diverse functions and could not be attributed to a single function. Of the 6 genes selective to *B. lata* strain 1299 four were also BIT selective.

In total 25 of 31 gene affecting variants are located in upstream or downstream genes and convey modifier effects which are unlikely to cause a change in core functions. 6 genes and 7 different experimental conditions had moderate or high impact on gene function. Benzyl alcohol preservative treatment was associated with 5/7 of these experimental conditions. Of the five genes associated with benzyl alcohol and moderate/high impacts, four variants were located in cell membrane constituents. Furthermore, adaptive resistance experiments using benzyl alcohol preservative failed to decrease preservative susceptibility from wild type levels in 7/8 experiments. This indicates that benzyl alcohol treatment causes damage to bacterial cell membranes which reduces adaptive resistance potential.
The two variants affecting *soxR* a transcriptional activator for inducing resistance to reactive oxide, both had moderate/high effects on the function. While the effect of the function of variants cannot be fully understood without further experimentation, this indicates that *soxR* functionality is not essential for bacterial adaption to preservatives.

In the analysis of the top 10% of variants shared amongst preservative treated Bcc strains, variants were observed in multiple genes involved in stress resistance. These genes were involved in various mechanisms including management of cell membrane permeability (*envZ, ompR, clcA*), induction of dormancy (*hrp1*), protection of cellular components (*hslO* and *htrA*), sensing hypoxia or high oxidation activity (*cmpR*), induction of mutagenesis (*dnaE2*) and the incorporation of foreign DNA (*comEC*). The observed variation in genes involved in multiple stress resistance mechanisms indicate that stress resistance is significant in adaptive bacterial response to preservatives.

Various genes were also associated with the degradation of toxins including ethanols (*acoD, hxlR*), ethylbenzene (*apc3*), benzoates (*benM*) and formaldehyde (*hxlR*). Variants were also seen in genes involved in the degradation of toxic polyamines

which are commonly generated from ethanol stress (*speG*, *puuA* and *puuC*). The variation affecting genes involved in multiple degradation pathways could demonstrate this is an important preservative resistance mechanism within preservative exposed Bcc strains.

Variants were seen affecting tripartite efflux pump components of *acrB*, *bepE*, *mexA, srpC* and *ttgR*. Interestingly, while three of these are well characterised in antimicrobial efflux (*acrB*, *bepE* and *mexA*) two are known to be involved in solvent efflux (*srpC and ttgR*) indicating that unlike traditional antimicrobial agents, preservative resistance response may involve solvent efflux activity.

Variants were observed in two genes concerning the cell membrane structure: *kdsA* and *sadH*. The variation affecting *kdsA* and *sadH* in Bcc strains exposed to preservatives demonstrates the importance of maintenance of the cell envelope structure and outer membrane biosynthesis to broad range preservative resistance.

Four genes were seen to be involved in cellular damage response, including repair of alkylation damage (*ada* and *alkA*), resistance to reactive chlorine species (*rclR*) and resistance to reactive oxygen species (*soxR*). Damage response seem to be selective, as *ada* and *alkA* are observed with BIT preservative exposed *B. lata* strain 1299 only while *rclR* is seen across all preservatives and evolution methods but in *B. cenocepacia* strains 1291 and 1292. The relative effect of *soxR* and superoxide resistance in Bcc bacterial adaption to preservatives is unclear due to the presence of high/moderate impact variants in both experimental conditions.

Variants were seen to be involved in biofilm mechanisms, including biofilm signalling (*gmr* and *mcbR*) and biofilm initiation (*lutP* and *sadA*). The observed variation affecting these genes indicates that the initiation of biofilm and its induction through signalling and transcriptional activation is important to *Burkholderia* species adaption and preservative resistance.

5.4.5 GO Enrichment

For the 18 samples with data, the total number of annotated topGO results ranged from 7-197 terms. However, the total number of significant GO terms for experimental conditions were much lower (Appendix 4). For the 19 samples, the Fishers statistical method and Classic/Elim, algorithms only gave significant GO terms for 6/7 experimental conditions respectively and the Weight and Weight01 algorithms only gave significant GO terms for 1/3 experimental conditions respectively. Therefore, analysis of significant GO terms associated with species and preservatives could not be performed.

On average the number of significantly annotated topGO terms were greater for L/S experiments (N= 9) than H/F experiments (N=9), for example with the Fishers statistical method and Classic algorithm, there were only 8 significant topGO terms for the H/F experiments however there was 109 significant topGO terms for the L/S experiments (Appendix 4).

There were 21 common GO terms seen across all four algorithms (Figure 34). All three GO Types are represented, there are 9 Biological Process (BP), 10 Molecular Function (MF) and two Cell Component (CC) terms (Table 29). Scatter plots for semantically similar GO terms within BP and MF GO types were assembled using REVIGO (Supek *et al*., 2011) and are displayed in figures 35 and 36 respectively.

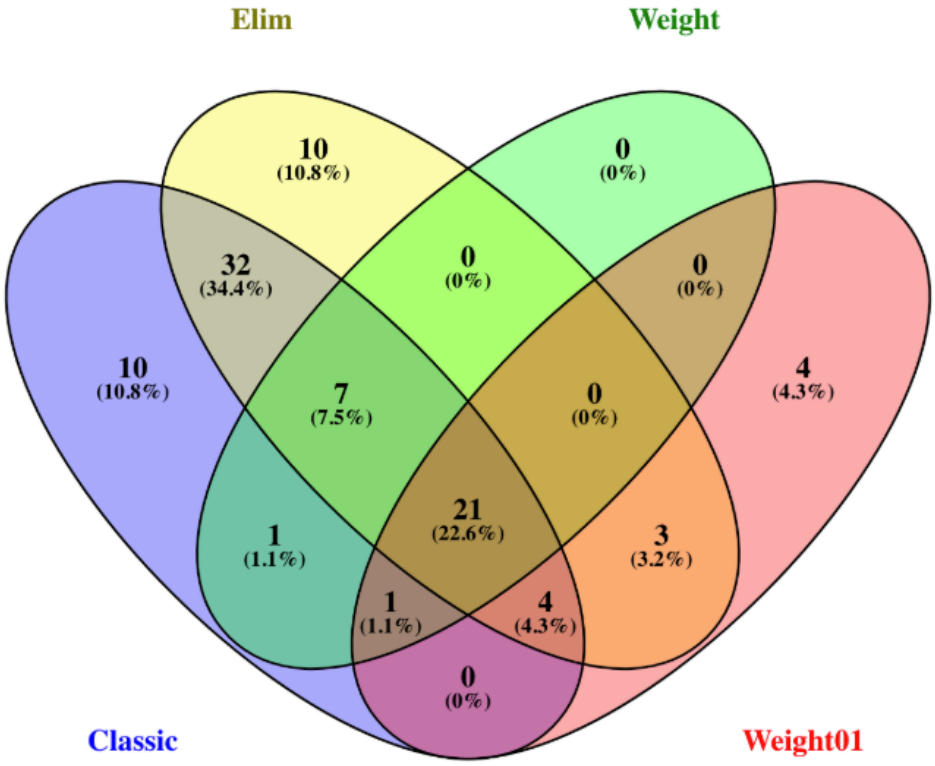


Figure 34: Common GO Terms shared with topGO package GO enrichment analysis for all experimental conditions using the fisher's statistical method and "Classic", "Elim", "Weight" and "Weight01 algorithms.

The 21 GO Terms are broad but can be roughly grouped into 5 functional categories:

1. Adaptive Resistance Response (3)
2. Cellular Components (3)
3. Transport (2)
4. Metabolism (3)
5. Xenobiotic Detoxification (10)

Adaptive Resistance Response

The GO term "cellular response to chemical stimulus" relates to changes in cellular biological processes that occur in response to a chemical stimulus (EBI, 2019). This is broad and can affect the state of the cell via changes in cellular activities such as locomotion, signalling, secretion, enzyme biosynthesis and gene expression (Ashburner *et al*., 2000). In this experiment essential biological processes are being

altered by an endogenous preservative substance and eliciting cellular changes as stress response and preservative resistance mechanisms are activated.

"Methylation" and "Methyltransferase Activity" are the process of attaching a methyl group to a molecule (EBI, 2019). Although methylation is a normal cellular process it is also considered to be key in epigenetic changes during adaptive resistance response. Methylation of DNA bases during bacterial adaption to antimicrobials is hypothesised to cause increased gene expression through increased transcription factor binding and inheritance of advantageous methylation patterns to subsequent generations (Lehmann *et al*., 1995).

Cellular Components

Of the 21 GO terms, only two are Cellular Component types: "Integral Component of Membrane" and "Cytoplasm" (EBI, 2019). The "integral component of membrane" concerns a gene and/or protein which has a proportion integrated into the hydrophobic tail of the phospholipid component of the membrane. The cellular membrane is the target for most biocides, making its active efflux, surface attachment (essential for biofilm) and enzymatic defences essential properties for biocide resistance (McDonnnell and Russell, 1999).

The "Cytoplasm" is the gel like matrix inside the bacterial cell and includes its important cell structures such ribosomes but not the cytoplasmic membrane or nucleoid (EBI, 2019). The cytoplasm is where integral cellular biological processes occur, such as metabolism and growth. Once inside the cell, biocides are detoxified and mineralised by various bacterial enzymes, although the exact metabolic process is unknown (Maillard, 2002). This is also indicated with location information of gene variants which are given in Tables 22-28. Of these 87 genes with variants from preservative exposed Bcc strains (13 associated with stable adapted resistance and 74 associated with all strains exposed to preservatives) 30% (26) were cell membrane associated, whilst 70% (60) were cytoplasm associated.

"Structural Constituent of Ribosome" is the action of a molecule that strengthens the structure of the ribosome (EBI, 2019). Maintenance of the structural integrity of the ribosome is important. Although biocides do not target ribosomes directly, the action

of biocides has been seen to cause ribosomal damage (Nakamura and Tamaoki, 1968; Miall and Walker, 1967; Wang and Matheson, 1966).

Transport

The BP GO term "Transport" and MF GO term "transporter activity" both relate to the translocation of objects such as macromolecules, small molecules, ions, either in uptake or expulsion via transporters and pores (EBI, 2019). A well characterized method of biocide resistance is efflux action of transporter proteins, by active or passive means to expel toxic biocide that exists in the periplasmic space (Gilbert and McBain, 2003).

Metabolism

The three metabolism-associated GO terms concern very different and unlinked processes ATP binding, transition metal ion binding and glutamine family amino acid metabolic process.

"ATP binding" is described as an interaction with adenosine 5'-triphosphate (EBI, 2019). Within all forms of life, ATP is universally important, responsible for energy metabolism via cellular respiration, used in various ATP coupled reactions, in cellular signalling via kinases, in cAMP synthesis and in the formation of nucleotides and other derivatives such as ADP AMP (Li, 2018). In bacteria ATP-binding is essential for the function of ATP-binding cassettes, which are multiunit transporters that rely on ATP binding and hydrolysis for the translocation of substances across the cellular membrane. ATP binding cassettes have broad biological functions but are important for pathogenicity, virulence and antimicrobial resistance (Lewis *et al*., 2012).

The "glutamine family amino acid metabolic process" encompasses any chemical pathways involving amino acids of the glutamine family: arginine, glutamate, glutamine and proline (EBI, 2019). The glutamine family of amino acids have molecular functions beyond their involvement in metabolism. In various bacterial species swarming motility is triggered when glutamate family amino acids are utilised as a primary nitrogen source. Swarming has been seen to assist in transient

resistance to antibiotics (Overhage *et al*., 2008; Kim and Surette, 2003, 2004; Kim *et al*., 2004; Köhler *et al*., 2000).

"Transition metal ion binding" is the interaction of a metal (such as iron or copper) in a transition state, an incomplete shell of atom electrons which results in cation (EBI, 2019). In bacterial metabolism metals are essential for various cellular processes. Various pathogenic species of *Burkholderia* including *B. cenocepacia* induce metal uptake in iron limited conditions such as the host environment (Yoder-Himes *et al*., 2009). In various stages of chronic infection development *B. cenocepacia* strains from CF patients upregulate 1000 genes involved in iron acquisition and other survival mechanisms such as translation, efflux and cellular adhesion (Mira *et al*., 2011). Transition metal ions lack a full d-shell of electrons. These metal ions therefore have beneficial catalytic properties and are important enzymatic co-factors but can also cause damage through their redox reactivity and ability to produce oxides (Palmer and Skaar, 2016). Whilst transition metal iron binding is a part of normal regulation of intercellular metal concentrations, it could be involved in binding reactive metals in preservative stressed Bcc cells which would result in the reduction of transition metal redox and inhibition of toxic oxygen radical production.

Xenobiotic Detoxification

This group contains four generalist terms for binding and breakdown: small molecule binding, oxidation-reduction process, oxidoreductase activity and hydrolase activity (EBI, 2019). The remaining six terms are more specific to the binding and catabolic process surrounding xenobiotics: organic cyclic compound binding, heterocyclic compound binding, benzene-containing compound metabolic process, monocarboxylic acid catabolic process, organic cyclic compound catabolic process and aromatic compound catabolic process (EBI, 2019). In the REVIGO similarity scatter plots these terms all cluster together strongly in both the BP and MF plots (Figures 35 and 36). The one or more GO terms in this group are seen across 7-9 experimental conditions dependent on method (Appendix 5).

In aromatic compound degradation, enzymes cleave the aromatic ring into non-aromatic intermediates for use in the krebs cycle which feeds electrons into the

electron transport chain (Díaz *et al*., 2001). A model for the bactericidal effect of antibiotics involves the hyperactivation of the electron transport chain or tricarboxylic acid cycle (TCA) which leads to damage of iron-sulphur clusters via the Fenton reaction, producing hydroxyl radicals (Kohanski *et al*., 2010). Interestingly *E. coli* and *S. aureus* mutants defective in elements necessary for respiratory chain and/or Fenton reaction show increased antibiotic resistance . Therefore, importance of aromatic compound degradation (as highlighted by GO enrichment) could serve to functionally reduce the TCA to decrease superoxide production and increase resistance.

Summary

Due to a lack of significant GO terms across all experiments using four algorithms, GO terms associated with species and preservatives could not be performed. However, the number of significantly annotated GO terms were larger for L/S experiments than H/F experiments. This was presumably caused by the higher number of variants associated the L/S method.

There were 21 GO terms seen across all four algorithms which could be condensed into 5 broad functional categories: Adaptive Resistance Response (3), Cellular Components (3), Transport (2), Metabolism (3) and Xenobiotic Detoxification (10).

The Adaptive Resistance Response GO terms “Cellular response to chemical stimulus”, “Methylation” and “Methyltransferase Activity” demonstrate the importance of global cell and protein regulation in mediating adaptive resistance responses to biocide.

The cellular component GO terms “Integral Component of Membrane” and “Cytoplasm” are likely to be highly significant in terms of preservative exposed *Burkholderia* strains as these organisms are eliciting cellular changes in integral components in response to preservative treatment which often target these components.

The GO terms involved in transport and metabolism are broad however these can be associated to mechanisms seen employed by bacterial species to confer antimicrobial resistance.

The majority of the GO terms were associated with Xenobiotic Detoxification. In the context of preservative resistance, unlike antimicrobial resistance it seems that detoxification of xenobiotic compounds is highly significant.

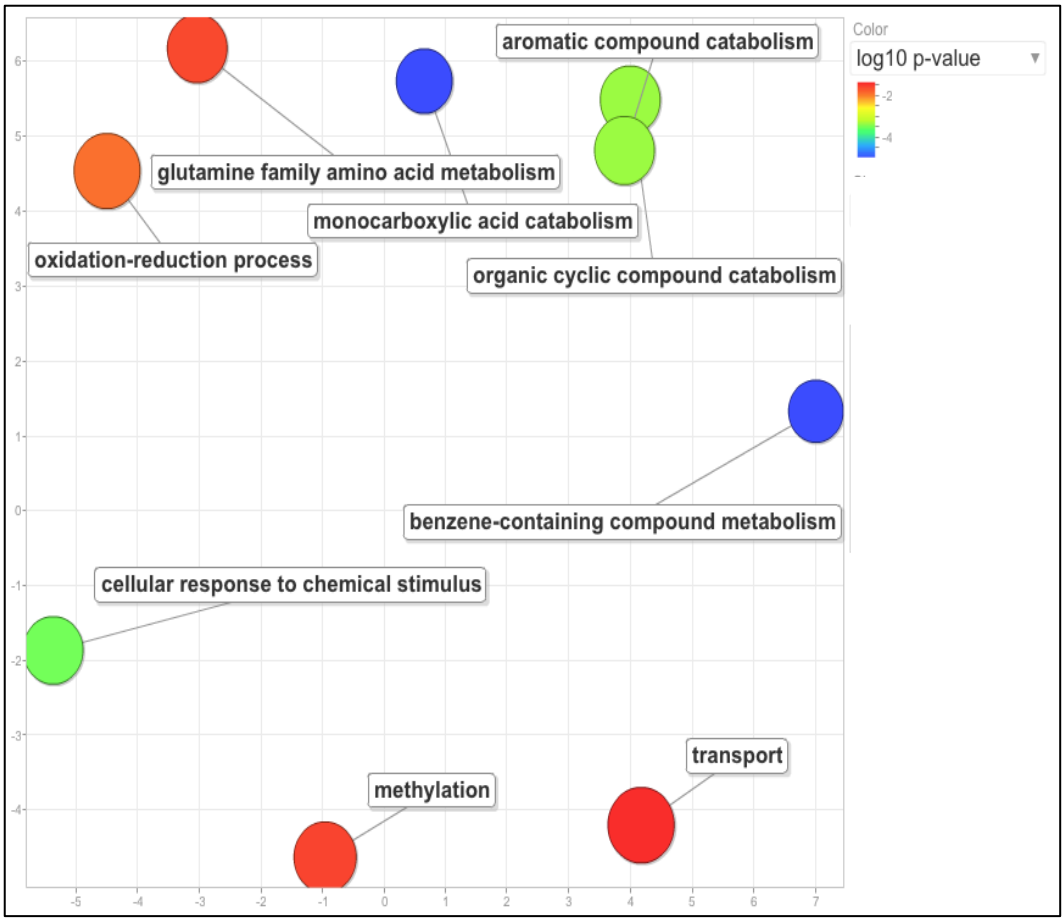


Figure 35: Scatterplot of 9 Biological Process (BP) GO Terms. Terms are clustered according similarity and coloured according to P value.

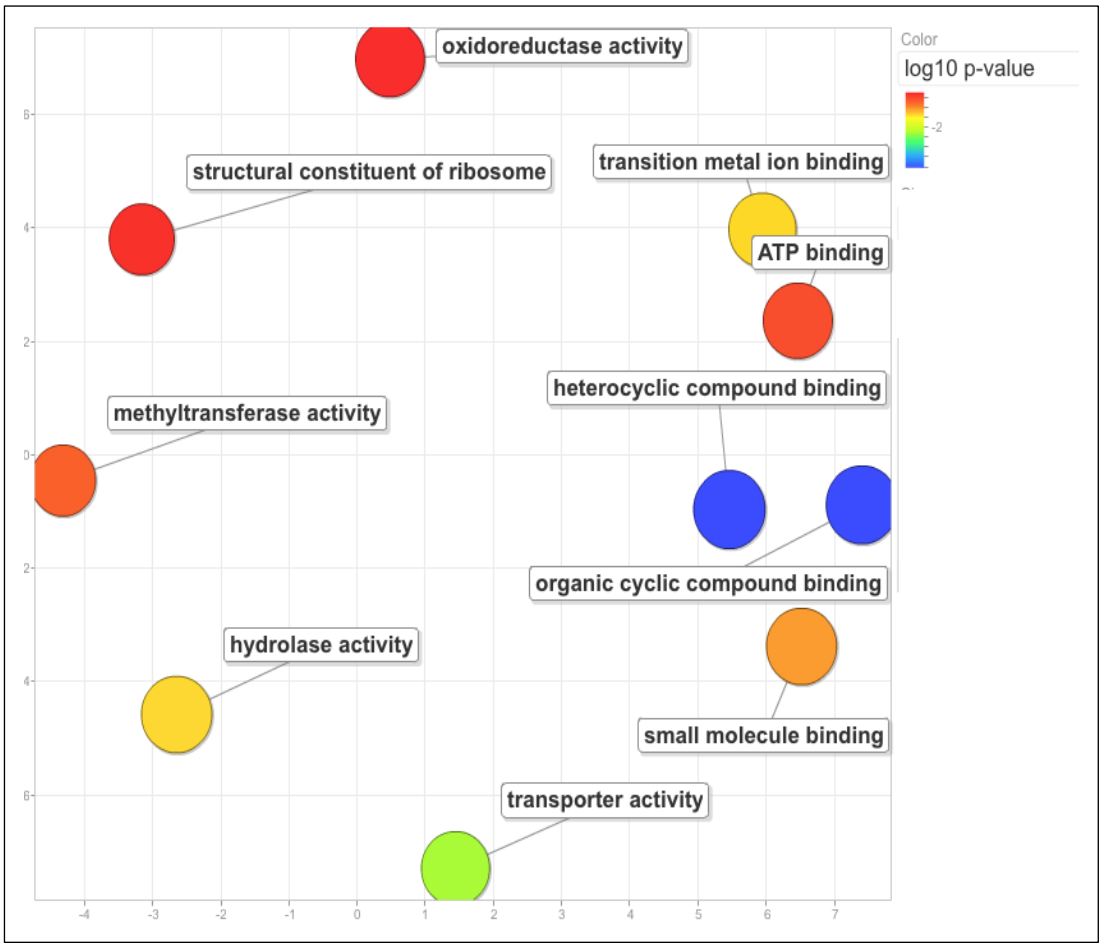


Figure 36: Scatterplot of 10 Molecular Function (MF) GO Terms. Terms are clustered according similarity and coloured according to P value.

5.5 Discussion

5.5.1 Variant Counts

Whilst the volume of variants in a given sample is not predictive of any functional genotype, it does demonstrate an increased selection pressure. This is supported through the presence of SNPs in *dnaE2*, an error prone DNA polymerase common in proteobacteria which encourages mutagenesis (Peng *et al*., 2017). Despite their potential to cause deleterious effects, bacterial populations where spontaneous mutagenesis is induced are seen to adapt quicker to environments (Sniegowski *et al*., 1997). Mutagenesis creates a larger allelic abundance in which beneficial traits can be selected. Natural selection then replicates and spreads the advantageous mutator allele in the bacterial population. Therefore, despite the cost of mutation, the potential of greater survival under unfavourable environmental conditions drives the mutational increase (Ilmjärv *et al*., 2017). Exposure to antibiotics and biocides are seen to cause an increase in frequency of mutation, horizontal gene transfer, plasmid and phage transference in bacterial populations (Wales and Davies, 2015).

The abundance of variants in H/F experiments compared to L/S experiments were significantly different. For H/F experiments, the average count of variants is lower than controls, while the average count of variants in L/S experiments was 39.6 times higher than controls (Figures 29 and 30). This was also seen with the GO enrichment analysis, where the L/S experiments had 13 times greater amount of significant GO terms than H/F experiments (Appendix 4). This follows the current prediction in literature, whereby adaptive resistance experiments utilizing a high concentration and fast exposure methodology result in transient resistance and are seen to engage transcriptional upregulation mechanism. Whereas adaptive resistance experiments utilizing a low concentration and slow exposure methodology result in mechanisms which result in stable resistance and are often the consequence of mutations and compensatory mechanisms (Motta and Aldana, 2016).

There was no correlation to variant count and fold changes in preservative susceptibility (Figures 31 A/B). Therefore, genotypic mutation abundance did not correlate to phenotypic preservative susceptibility. While mutation abundance is

associated with resistant antimicrobial genotypes, hypermutation in non-clinical *Burkholderia* species is low, despite their broad antimicrobial resistance characteristics (Martina *et al*., 2014). Other mechanisms of adaptive resistance such as transcriptional changes, could be a better predictor of potential bacterial adaptive resistance to preservatives.

In this study, variant counts (without controls) seen in more than one experimental condition, were higher for BIT than for phenoxyethanol or benzyl alcohol (Appendix 2). Phenoxyethanol and benzyl alcohol are classed as eco preservatives, due to their natural composition whereas BIT is a synthetic compound. A hypothesis for the increased mutation abundance associated with BIT is the lack of natural resistance mechanisms which encourages sporadic mutagenesis whereas alcohol-based benzyl alcohol and phenoxyethanol have natural homologues and are more likely to induce an upregulation of existing resistance pathways. A recent study into gene expression for *P. aeruginosa* exposed to phenoxyethanol and BIT preservatives found gene expression was higher for phenoxyethanol than BIT (Green *et al*., 2018).

5.5.2 Biological Function of Variants

In this study, investigation of how the variant may affect the protein function is purely putative and gained from snpEFF and topGO analysis. It is difficult to assess the impact of mutations without further experimental investigation using directed mutagenesis and complementation studies to demonstrate their functional contribution to preservative induced adaptive resistance of Bcc strains (Jahn *et al*., 2017; Sandberg *et al*., 2014; Lieberman *et al*., 2011; Yang *et al*., 2011).

5.5.3 Stable Adaptive Resistance

The function of the 13 genetic mutants seen in *Burkholderia* strains which developed stable preservative-induced adaptive resistance were not associated with traditional antimicrobial resistance mechanisms but were seen in general mechanisms of stress response, signalling, metabolism and enzymatic degradation. Adaptive resistance to multi-targeting antimicrobial agents is thought to be multifactorial and a reflection of the broad mode of bactericidal action (Fernández *et al*., 2011). Bacteria use a wide array of different physiological and genetic adaptions to mitigate damage and enhance survival. This differs from antibiotic agents which often have a singular cellular target (Wales and Davies, 2015). The relevance of these general mechanisms

of stress response, signalling, enzymatic degradation and metabolism are discussed in Section 5.5.4.2.

5.5.3.1 Effects of Preservative, Species/Strain and Method type on Stable Adaptive Resistance Variants

Genomic evidence for stable adaptive resistance mechanisms is lacking. In this study Bcc strains gained stable adaptive resistance to the preservatives phenoxyethanol and BIT in both high gradients with short exposure timeframes (H/F) and low gradient with long exposure timeframes (L/S). However, the fold change in preservative susceptibility was greater for long exposure and low gradient experiments. In this experiment variant counts were observed to be higher for L/S experiments when compared to H/F experiments. This is also seen in analysis of stable adaptive resistance associated variants where of the 13 gene variants seen in strains that developed stable adaptive resistance, the majority (10/13) were from L/S experiments. This demonstrates that high level stable adaptive resistance generated from long exposure and low gradient experiments (L/S) is associated with genomic variants.

In the analysis of stable preservative induced adaptive resistance three gene variants seen in short-exposure and high gradients experiments (H/F) all occurred in adaptive resistance experiments using the preservative phenoxyethanol. This demonstrates that the preservative phenoxyethanol in short timeframes and high gradients is able to induce genetic traits associated with long exposure and low gradients (L/S) and indicates this preservative has a greater adaptive resistance potential.

In this study 12 of the 13 gene variants seen only in stable adaptive resistance experiments and in more than one experimental condition could not be correlated with species/strains and preservative. Only one gene was selectively associated, the BIT unique gene *cqsA* which is a synthase of the quorum-sensing autoinducer CAI-1. First described in *Vibrio cholerae*, *cqsA* (*cholera*e quorum-sensing autoinducer) has been found in various *Vibro* spp. (Lorenz *et al*., 2017). In *Vibro* species, the quorum-sensing autoinducer CAI-1 is sensed by *cqsS*. This two component QS signalling system regulates a collection of genes for bioluminescence, a type III secretion system, and metabolically significant proteins such as metalloprotease (Henke and Bassler, 2004). A homologue of the CAI-1 and *CqsS* is present in

Burkholderia xenovorans, genes *bsqA* and *bsqS*. This signalling system controls various bacterial virulence factors including biofilm synthesis, formation of extracellular filaments, and genomic island expression (Tiaden *et al*., 2010). Sensory regulators such as two component QS signalling systems enable adaptive responses to changes in environmental conditions and are important for survival and host colonisation. This QS signalling gene variant associated with BIT preservative selectively indicates that QS signalling of bacterial survival virulence factors is involved in BIT preservative induced stable adaptive resistance.

5.5.4 Preservative Exposed Bcc Strains

There was only small number of variants exclusive to Bcc strains which demonstrated preservative induced stable adaptive resistance and were not found in controls or organisms which did not demonstrate resistance or demonstrated transient resistance. However, it is known that certain intrinsic resistance mechanisms are also associated with adaptive resistance mechanisms for example porin size and expression, efflux activity and enzymatic degradation (Coenye and Mahenthiralingam, 2014). Therefore, the putative functions of gene variants of preservative exposed *Burkholderia* strains was also examined.

5.5.4.1 Effects of Preservative, Species/Strain and Method

L/S and H/F Methodology

Motta and Aldana (2016) hypothesized that slow and stable mechanisms (SSM) of adaptive resistance which are induced through long exposure timescale enable the development of compensatory mechanisms for growth handicaps associated with antimicrobial resistance. SSM mechanisms are thought to confer stable adaptive resistance. In this study, two methodologies were designed: A high and fast (H/F) protocol using the highest concentrations of preservative in fast successive passages (24-48 hours) and a the slow and low (S/L) protocol using sub-inhibitory concentrations in slow passages (24-72 hours). In the examination of 31 gene variants seen in preservative exposed Bcc strains, 12 were seen in L/S experiments while only 2 were seen in H/F experiments only. The majority of these genes concerned stress resistance. Stress response mechanisms enable bacteria to thrive in adverse physical and chemical stressors including antimicrobial treatment. Research suggests that in early exposure to antibiotics, stress responses are essential to

mediate antibiotic stress (Eswara and Kumavath, 2017). Therefore, it is likely that long exposure to sub-inhibitory concentration gradients of preservatives enables the development of compensatory stress mechanisms to cope with the biocidal effects of preservative agents.

Little is known about fast and transient mechanisms (FTM) of adaptive resistance characterised by a fast timescale however it is hypothesised that small epigenetic changes that leads to transient phenotypic resistance (Motta and Aldana, 2016). In this study, the two genes observed in H/F experiments only are both seen to contribute to the degradation of toxic substances. *Burkholderias* ability to degrade toxic organic compounds enables it to inhabit diverse ecological niches too toxic for many bacterial species (Choi *et al*., 2013). Some studies have demonstrated *Burkholderia* strains with increased resistance to antibiotics also have increased aromatic compound degradation capabilities (Loutet *et al*., 2011; Sass *et al*., 2011). It has been hypothesised that *Burkholderia* species have diverse metabolic capacities could facilitate modification or degradation of antibiotics and biocides through the action of general degradation enzymes (Coenye and Mahenthiralingam, 2014). This indicates that toxic substance metabolism could be significant for preservative degradation and cellular survival during short exposure to high concentrations of preservatives.

BIT

The four genes associated with BIT preservative were also *B. lata* 1299 strain type selective. Furthermore, this strain exhibited exceptional BIT resistance (See section 4.4.1 Preservative susceptibility of a panel of *Burkholderia* strains to nature identical and synthetic preservatives systems). This indicates that these genes are involved in increased resistance to BIT preservative agents. The isothiazolinone class of preservative agents are potent oxidizers which act on protein thiols of cysteine residues (Halla *et al*., 2018). Three of the four genes associated with BIT preservatives were involved in damage response: *ada/alkA* involved in alkylation damage and *speG* involved in the degradation of toxic polyamines which accumulate under cellular oxidation. The significant of alkylation repair which has not previously been seen in antimicrobial resistance is hypothesised here to be the repair of DNA damaged cysteine-alky groups. The degradation of toxic polyamines has

been observed during bacterial oxidative stress responses. Transcriptional analysis of *Burkholderia pseudomallei* during oxidative stress has shown *speG* is regulated by sigma factor E which also controls other oxidation responses (Jitprasutwit *et al*., 2014). The fourth gene, *lutP* is involved in biofilm synthesis, which is known to offer greater oxidisation protection due to its complex matrix structure. In *Burkholderia cenocepacia* biofilms, treatment with a QS inhibitor results in impaired oxidative stress responses resulting in increased sensitivity to antibiotics (Slachmuylders *et al*., 2018). It is assumed then that damage/oxidation resistance mechanisms are central to BIT resistance in *B. lata* strain 1299.

Benzyl Alcohol

In this study preservative specific mutations for benzyl alcohol was associated with efflux: pump systems. Furthermore, another efflux pump gene variant is also seen with four benzyl alcohol preservative experiments and one phenoxyethanol experiment. Benzyl alcohol preservative was also associated with the majority of moderate/high impact mutations. Of the 5 genes associated with benzyl alcohol and moderate/high impact mutations, four were cellular membrane components (*kdsA, ompR, mexA and acrB*). At low concentrations, alcohols such as benzyl alcohol and phenoxyethanol act on bacterial membranes to cause damage leading to cell lysis (Halla *et al*., 2018). Benzyl alcohol has even been observed to attack outer membrane proteins such as efflux pumps (Yano *et al*., 2016). In adaptive resistance experiments, decreased preservative susceptibility to benzyl alcohol preservative of *B. cenocepacia* strains could not be induced in all experiments and was only transiently increased in *B. lata* strain 1299 L/S experiments. This indicates the bactericidal effect of benzyl alcohol seems to be caused by significant damage to cellular membrane components including efflux pump constituents which leads to cell death.

Species/Strain Specific Effects

Species associated mutations were not observed for *B. cenocepacia* and could not be asserted for *B. lata* due to the lack of multiple strains. Weak strain associations were seen for gene variants, with 6 genes clustered by *B. lata* 1299 strain type (however four of these were also BIT selective), 3 gene variants associated with *B. cenocepacia* strain 1291 and two genes variants associated with *B. cenocepacia*

strain 1318. Strain associated gene variants in preservative exposed Bcc strains was observed to be with functionally diverse genes. Therefore, it is difficult to understand the advantage given to these strains. In Chapter 4 (see section 4.4.1 Preservative susceptibility of a panel of *Burkholderia* strains to nature identical and synthetic preservatives systems), species associated decreases in preservative susceptibility could not be identified due to the large variability in strain susceptibility. Strain differences have also been identified in Bcc bacteria preservative resistance previously (Rushton *et al*., 2013).

5.5.4.2 Putative Functions of Gene Variants of Preservative Exposed Bcc Strains

These mechanisms have been grouped loosely into 8 core functional categories subjects:

1. Stress resistance
2. Degradation of toxins
3. Efflux pump systems
4. Cellular membrane
5. Damage response
6. Biofilm
7. Signalling
8. Metabolism

Stress Resistance

In the analysis of the top 10% of variants shared amongst preservative exposed Bcc strains, genes were associated with various stressors. Environmental stress induces global changes in cellular activities such as gene expression and cellular states. Global changes in cell physiology are seen in this experiment in the GO enrichment analysis as signified by “the cellular response to chemical stimulus” GO term.

Often stress resistance mechanisms overlap with antimicrobial resistance mechanisms such as the induction of active efflux (Poole, 2014), changes to outer membrane barrier structure (Miller, 2016), detoxification of toxic by-products (Tkachenko *et al*., 2012) and promotion of resistant biofilm structures (Singh *et al*.,

2017). In this experiment variants were also seen in genes controlling efflux, outer membrane architecture, detoxification and biofilm synthesis.

In this study GO enrichment and gene variants indicate that five main types of stresses affect preservative exposed Bcc species: Osmotic stress, Oxidative stress, Nutritional Stress, Evolution stress, and Ribosomal stress.

Osmotic Stress

Preservatives are known to act on bacterial cell membranes which causes loss of cell membrane structure integrity and leaching of cell components resulting in osmotic shifts (Oh *et al*., 1998). In this experiment variants were seen in three genes associated with osmotic stress (*envZ/ompR, htrA*). GO enrichment analysis results also suggested osmotic effects may be affecting preservative stressed Bcc strains. The "glutamine family amino acid metabolic process" includes any biological process involving amino acids of the glutamine family such as glutamate. A metabolic profiling study of osmolarity response of five closely related *B. cenocepacia* strains found that osmotic stress tolerance was attributed to high cellular concentrations of various amino acids including glutamate. It was therefore hypothesised that these metabolites acted as osmoprotectants and were utilised by *B. cenocepacia* as a strategy to restore osmotic gradients (Behrends *et al*., 2011). Therefore, osmotic stress response and restoring osmotic homeostasis is essential for cellular survival when exposed to preservatives.

Oxidative Stress

In this study variants were seen in genes concerning oxidative stress resistance (*surE*), repairing/removing oxidatively damaged proteins (*hslO* and *htrA*), removing toxic by-products formed from oxidative stress/damage (*gpr, speG, puuA, and puuC*), sensors of oxidising agents (*srrA*) and transcriptional activators of oxidative damage response pathways (*rclR* and *soxR*). Resistance to oxidative stress and oxidative DNA/protein damage is important for *Burkholderia* preservative survival as many preservatives induce oxidative-mediated cell death (Kohanski *et al*., 2010). The ability of Bcc biofilms to resist oxidative cell death has been observed and is thought to be caused by various damage response mechanisms such as an upregulation of general stress responses, specific oxidative stress responses and

synthesis of proteins involved in repair of cellular damage (Peeters *et al*., 2013). A study looking at global gene expression in two *B. cenocepacia* strain J2315, with one isolate displaying MDR found an upregulation of a six gene cluster containing putative functions in resistance to DNA damage caused by reactive oxygen species in both organisms (Sass *et al*., 2011). Therefore, variants in oxidative stress resistance likely play an active role in antimicrobial resistance in Bcc strains.

Nutrient Stress

In this experiment variants were seen in genes *rsh* and *rplK* which control the activation of dormancy response through the alarmone guanosine tetra/penta phosphate or (p)ppGpp. A variant is also seen in *hrp1*, a hypoxic response protein which induces dormancy. In response to stress bacterial cells decrease metabolic activity and cell division. This in turn decreases antimicrobial efficacy as these only work on metabolically active cells (Miller *et al*., 2004; Eng *et al*., 1991). By shifting metabolic states into dormancy vegetative variants of a metabolically active bacterial population form a persister cell sub-population. Persister cell sub populations are able to restore growth and metabolism as this is not caused by mutation. In pathogenic *Burkholderia* strains, post antibiotic treatment recurrent infections are common and are presumed to be caused by antibiotic tolerant persister sub populations (Nierman *et al*., 2015). While it is thought that general mechanisms of stress protection and repair cause indirect biocide/antibiotic cross resistance, evidence suggests that the persister sub population may be more directly involved (Khakimova *et al*., 2013; Orman and Brynildsen, 2013). One study found *E. coli* tolerance to antibiotics is mediated by an active starvation response as opposed to passive growth arrest (Nguyen *et al*., 2011). The reduction in cellular function is also seen with the variant in *srrA,* which is known to represses virulence factors in response to low oxygen including reducing expression of *agr* (accessory gene regulator) effectors, production of TSST-1 exotoxin, and the surface associated virulence factor protein A (McCormick *et al*., 2003). In *B. dolosa*, a similar oxygen-sensing two-component system controls various mechanisms for bacterial survival and virulence including biofilm, intracellular invasion, persistence and motility (Lehmann *et al*., 1995).

Although, these genes are key in activating stringent response these are also involved in other stress functions. (p)ppGpp alarmone is also seen in cellular stress signaling to various stressors including initiating virulence factors such as type IV secretion system (Gaca *et al*., 2015). Overexpression of *Hrp1* in infection models is seen to enhance *Mycobacterium* survival responses to host immune defences and increased host cell death (Sun *et al*., 2017).

In the GO enrichment "transition metal ion binding" and "aromatic compound catabolic process" were both significantly enriched. Kohanski *et al*. hypothesised that cellular death from various bacteriacidal agents is caused by the hyperactivation of the electron transport chain in the TCA cycle resulting in super oxide damage to proteins which release ferrous iron which is oxidised to form hydroxyl radicals via the Fenton reaction (Kohanski *et al*., 2010). In *E. coli* strains under long term hydroxyurea stress increased iron uptake which triggered the creation of superoxide and led to cell death. (Wales and Davies, 2015). Mutations in elements associated with the respiratory chain, oxidative stress and iron metabolic networks greatly decrease antibiotic susceptibility and cell death (Yeom *et al*., 2010; Kukora and Helling, 1971). Transcriptomic analysis of an antibiotic tolerant persister cells sub-population of *B. cenocepacia* demonstrated downregulation of several genes from the TCA cycle and electron transport chain along with upregulation of carbon metabolism through the glyoxylate cycle in order to prevent ROS generation (Acker *et al*., 2013). Therefore, the GO enrichment results suggest that arrest of the TCA cycle (through dormancy) plays a role in increasing preservative resistance in Bcc strains through reduction of metabolic pathways contributing to superoxide production.

Evolution Stress

In this study of preservative treated Bcc strains there was a variant in *dnaE* which is associated with stress-induced mutagenesis. Activation of stress responses such as the SOS response commonly trigger the activation of error-prone DNA polymerases. Error-prone DNA polymerases can replicate from damaged DNA but can also act on undamaged DNA which then introduces mutations. SOS response driven mutagenesis induced by various antimicrobial agents has been described in *E. coli*, *S. thyphimurium*, *S. aureus*, *Mycobacterium fortuitum*, *Streptococcus pneumonia* and

Vibrio cholera (Seier-Petersen *et al*., 2014). In various examples, SOS dependent responses were attributed to the generation of mutations which resulted in antibiotic resistant phenotypes (Thi *et al*., 2011; Mesak *et al*., 2008; Cirz *et al*., 2005, 2006). It is likely that in response to preservative stress, stress induced mutagenesis is triggered, increasing the spontaneous mutation rate to give rise to potential advantageous mutations.

In this study a variant was seen in a *comEC,* a natural competency and DNA uptake cytoplasmic membrane protein. *comEC* driven competency has been demonstrated in the pathogenic *Burkholderia*, *B. thailandensis* and *B. pseudomallei* (Heacock-Kang *et al*., 2018). Natural competency in seen in 60 bacterial species and is thought to contribute to acquired antibiotic resistance in bacteria (Domingues *et al*., 2012) It is likely that preservative exposure induces a stress response which increases genomic plasticity such as competence, in the hope of acquiring genomic DNA advantageous for survival of the bacterial population.

Ribosome Stress

The ribosome is an essential part of machinery to the cell, and bacteria have developed several ways to protect it from damage and stress. Compensatory mechanisms of ribosome stress include *mexXY-oprM* efflux induction, heat shock mediated protein responses, oxidative stress responses, and ppGpp alarmone signalling (Poole, 2012). In this study variants were seen in, *mexAB-oprM* efflux, heat shock protein chaperones *hslO* and *htrA*, ROS and RCS oxidative response initiators *roxS* and *rclR,* and ppGpp alarmone genes *rsh* and *rplK*. Ribosomal stress resistance is further indicated in the GO enrichment results. The GO term “Structural Constituent of Ribosome” indicates the molecular strengthening of the ribosome structure.

Degradation of Toxins

In this study variants are seen in enzymes *iorA/B*, *acoD, apc3, benM* and *hxlR* involved in degrading various toxic substances and in constituents of solvent efflux pumps *srpC* and *ttgR*. These variants are strongly associated with Bcc strains exposed to alcohol-based preservatives benzyl alcohol (10), phenoxyethanol (5) and less so with the isothiazolinone preservative BIT (3). This could be caused by the

similarity in compound structure to benzoic acid. Benzoic acid ($C_7H_6O_2$) and benzyl alcohol ($C_6H_5CH_2OH$) are related compounds, with very similar aromatic ring structures, however benzoic acid has a carboxyl group (COOH) while benzyl Alcohol has the hydroxyl group (OH). *Burkholderia xenovorans* LB400 is known for its ability to hydrolse benzoates (salts and esters of benzoic acid and has three putative benzoate degradation pathways; *ben*, *cat* and *box* (Denef *et al*., 2004, 2005, 2006).

Interestingly, during exposure to aromatic compound *B. xenovorans* LB400 is seen to upregulate *GroEL* and *DnaK* molecular chaperones in response to the oxidative stress of these compounds (Martínez *et al*., 2007; Parnell *et al*., 2006). Oxidative stress responses to preservatives are also seen here including the presence of variants in two molecular chaperones *htrA* and *hslO*. There is further evidence of the involvement of alcohol-based detoxification mechanisms with various genes involved in the breakdown of toxic polyamines such as spermidine and putrescine which are attributed to a variety of extreme stress conditions including exposure to ethanol (Carper *et al*., 1991).

Variants associated with degradation/detoxification of toxic substances are also seen across both H/F (8) and L/S (10) experiments, suggesting it is important for both intrinsic and adaptive resistance. GO enrichment analysis results suggested degradation of toxic substances may be used as a resistance mechanism of preservative stressed Bcc strains. Enriched GO terms for binding and catabolic processes involved in xenobiotic detoxification include organic cyclic compound binding, heterocyclic compound binding, benzene-containing compound metabolic process, monocarboxylic acid catabolic process, organic cyclic compound catabolic process and aromatic compound catabolic process.

Many gram-negative bacteria are able to biodegrade xenobiotic pollutants into harmless by-products (Valderrama-Rincon *et al*., 2012). A number of environmentally isolated *Burkholderia* can degrade toxic substances and various common environmental contaminants such as insecticides, fungicide, herbicide, pesticides (Satapute and Kaliwal, 2016; Han *et al*., 2014; Lim *et al*., 2012; Poh *et al*., 2001), polycyclic aromatic hydrocarbons (PAHs) (Revathy *et al*., 2015; Suárez-

Moreno *et al*., 2011; O'Sullivan and Mahenthiralingam, 2005), monocyclic aromatic hydrocarbons (MAHs) benzene, toluene, ethylbenzene, xylene (BTEX) (Lee *et al*., 2016; Kahng *et al*., 2001) and industrial organic solvents (trichloroethylene and poly-chlorinated biphenyls (PCBs); (Parnell *et al*., 2006). The ability to remove toxic substances is attributed to the expression of enzymes able to oxidize and cleave aromatic compounds. Furthermore, some species are able to utilise pollutants for various cellular metabolic processes such as carbon cycling, nitrogen cycling and the electron transport chain (van der Meer, 2006; Roger *et al*., 1995).

In bacteria the enzyme mediated degradation of various biocides has been described for metallic salts (Cloete, 2003), parabens (Valkova *et al*., 2002) aldehydes (Kümmerle *et al*., 1996), quaternary ammonium compounds (QACs) (Nishihara *et al*. 2000) and phenols such as triclosan (Hundt *et al*., 2000). However, at present evidence is limited to environmental strains and has not been demonstrated in clinical strains.

Efflux Pumps

Variants are seen in five efflux pump components (*acrB, bepE, mexA, srpC* and *ttgR*) and across 16 experimental conditions. There is a strong association for benzyl alcohol preservative (12), rather than phenoxyethanol (3) or BIT (1) and a greater association with L/S (10) experimental conditions rather than H/F (6). This suggests active efflux of preservatives is more common with adaptive resistance than intrinsic resistance, and with the eco-preservative benzyl alcohol. GO enrichment analysis results also suggested transport may be used as a resistance mechanism of preservative stressed Bcc strains with the GO terms "transporter activity" and "transport" both enriched.

Efflux pumps are three component systems comprised of a cytoplasmic associated inner membrane protein (IMP), an associated periplasmic membrane fusion protein (MFP) connecting the inner and outer membranes, and a channel forming outer membrane factor (OMF) (Du *et al*., 2018). These protein complex structures are able to span the inner membrane, cytoplasm and outer membrane, making them highly efficient at extruding toxic substances such as antibiotics and solvents(Li *et al*., 2015) . In *Burkholderia* efflux pumps have been widely described as a mechanism

for expulsion of various antimicrobial compounds including preservatives (Coenye and Mahenthiralingam, 2014). Transcriptomic analysis of a Unilever industrial isolate *B. lata* strain 383 (LMG 22485), which was stepwise adapted to the preservative methylisothiazolinone-chloromethylisothia- zolinone (M-CMIT) found efflux pumps mediate innate preservative resistance. The *B. lata* 383 M-CMIT adapted strain exhibited a four-fold increase from MIC than wild type levels and demonstrated cross resistance to fluoroquinolone antibiotics. Increased gene expression was seen in three of the five efflux pump families the ABC superfamily, MFS family and RND family. Preservative susceptibility was restored using the efflux pump inhibitor L-Phe-Arg-beta-napthylamide (Rushton *et al*., 2013).

In this study variants were seen in the gene *bepE*. The *bep* efflux transporter are highly involved in toxic compound and multidrug efflux in *Brucella suis.* The *bepCDE* efflux pump is crucial to toxic compound efflux and can expel a broad variety of compounds such as antimicrobials, detergents, dyes and intercalators. Furthermore, activation of *bepCDE* in host survival is greatly induced, demonstrating its virulence importance (Posadas *et al*., 2007). Although *bep* are not seen in other gram-negative organisms, there is a high sequence homology with other important examples of RND type inner membrane proteins, *acrB* in *E. coli* and *mexB* from *P. aeruginosa. (*Martin *et al*., 2009).

Variants were also seen in *acrB*, of the RND *acrAB-tolC* efflux transporter system. This efflux complex is seen in gram-negative clinically relevant pathogenic bacterial species, such as *E. coli*, *S. enterica*, *K. pneumoniae*, and *E. cloacae* and has homologue systems in other gram-negative species (Veleba *et al*., 2013; Hentschke *et al*., 2010; Keeney *et al*., 2008; Ruzin *et al*., 2005). This efflux pump has a broad substrate specificity and confers resistance to multiple antibiotics, as well as dyes, organic solvents, detergents, disinfectants and biocides (Pérez *et al*., 2012). In a polymyxin-B sensitive strain of *B. vietnamiensis*, sensitivity was attributed to a mutation in an *acrAB* homologue called *norM* (Fehlner-Gardiner and Valvano, 2002).

In this study variants were seen in the membrane fusion protein *mexA*, of the well characterised *MexAB-OprM* RND type efflux system of *Pseudomonas*. *MexAB-*

OprM expression is not tightly regulated like other efflux systems and is always present in cells which is concurrent with evidence that *MexAB-OprM* are highly involved in intrinsic multidrug efflux (Chuanchuen *et al*., 2002). Many of the RND multidrug efflux pumps of *Burkholderia* are poorly/partially characterised, however a homologue of *mexA* is seen in *B. cepacia* (formerly *P. cepacia*), called *ceoA* which forms part of the *CeoAB-OpcM* multidrug resistance efflux pump (Podnecky *et al*., 2015). Another homologue of *mexA* is seen in *B. vietnamiensis* called *amrA* which is part of the *AmrAB-OprA* efflux transporter complex (Moore *et al*., 1999). The *mex* efflux pumps in *Pseudomonas* share a 50–71% homology with RND efflux pumps in *Burkholderia* (Buroni *et al*., 2009; Guglierame *et al*., 2006). Much of the research on efflux mediated resistance in *Burkholderia* species has centred around the pathogenic species strains *B. thailandensis* and *B. pseudomallei* and their antibiotic efflux capabilities. The *CeoAB-OpcM* complex of *B. cenocepacia* is seen to provide resistance to antibiotics chloramphenicol, fluoroquinolones and trimethoprim (Nair *et al*., 2004; Li *et al*., 1995). *Mex* RND type efflux pumps may be of importance in preservative resistance also. A recent study investigating differential gene expression of a Unilever industrial contaminant strain of *P. aeruginosa* on exposure to the preservatives BIT and phenoxyethanol and laundry detergent found *MexPQ-OpmE* efflux pump was repeatedly upregulated in all three conditions (Green *et al*., 2018; Nair *et al*., 2004).

In this study variants are seen in two components involved in solvent efflux, a transcriptional regulator of the ATP dependent solvent transporter system *ttg* and in the outer membrane protein of the RND type solvent efflux pump *srp*. The efflux pump *SrpABC* in the organism *Pseudomonas putida strain S12* confers solvent resistance to a broad range of organic solvents including hexane, toluene and dimethyl phthalate (Kieboom *et al*., 1998). The expression of this efflux system is induced when exposed to organic solvents and alcohol and is central to innate organic solvent tolerance in this organism (Kieboom and de Bont, 2001). The *srpABC* solvent efflux pump in the organism *Pseudomonas putida S12* is a homologue of the *ttgGHI* toluene efflux pump in *Pseudomonas putida DOT-T1E* (Yao *et al*., 2017). Transcriptional control of *ttgABC* via the *ttgR* transcriptional repressor is very important to resistance to both solvents and antibiotics. These efflux pumps are present without any environmental pressure and contribute to

innate resistance to toxic substances (Ramos *et al*., 1998). *TtgR* knock outs in *P. putida* DOT-TE1 resulted in overexpression of the *ttgABC* efflux pump, and a greater resistance phenotype (compared to the wild-type strain) to chloramphenicol, nalidixic acid, and tetracycline (Rojas *et al*., 2001).

Cellular Membrane

In this study, variants are seen in genes affecting Lipid A maturation in cell membrane biosynthesis (*kdsA*). In pathogenic *Burkholderia pseudomallei*, nutrient starvation resulted in upregulation of genes concerning energy metabolism and membrane biosynthesis, including *kdsA*. Mutants in cell membrane biosynthesis could not be constructed demonstrating these genes are essential for not only preservative viability but viability as a whole (Moore *et al*., 2008). Variants are also seen to affect *sadH* which is involved in the lipopolysaccharide organisation of the cellular envelope. In *Mycobacterium tuberculosis sadA/mymA* is seen to affect the composition of the long chain fatty acids in the cell envelope which affords protection against acid stress conditions, as well as increasing resistance to tuberculosis drugs, detergents and promoting invitro host survival under acidic pH (Singh *et al*., 2005). Enhancing and repairing the cellular membrane barrier is paramount for antimicrobial resistance. Although most biocides are multi-targeting, a common target is the cell membrane (Maillard, 2002). Damage to the cell membrane can cause disruption to osmotic gradients, leaching of cellular components and to increase permeability to allow more biocide to accumulate in the cystol. Effects of antimicrobial compounds once in the cystol are varied and can cause damage to organelles and denaturing of proteins, enzymes and DNA (Wales and Davies, 2015). Changes in outer membrane composition, including proteins, fatty acids and phospholipids have also been seen to decrease biocide susceptibility. Protection of the cell envelope and cell membrane structures occurs by the insertion of fatty acids which enables the repair against damage and is paramount to prevent cell lysis (Morente *et al*., 2013). In this study, variants are seen in genes *kdsA* and *sadH* which demonstrates the importance of cell membrane integrity and architecture to preservative resistance in Bcc strains.

Bacteria can also decrease the expression, size and activity of protein channels in the cellular membrane to decrease the permeability thus reduce antimicrobial entry

(Coenye and Mahenthiralingam, 2014). Outer membrane mediated reduction in diffusion and thus susceptibility has been demonstrated for clinical Bcc strains and various antibiotics. Bcc strain changes in membrane permeability by the preservative and common antibacterial personal care ingredient salicylic acid was due to decreased activity of the *OpcS* outer membrane porin (Burns and Clark, 1992). In this study, variants were seen in the outer membrane porin control system *envZ/ompR.* When intracellular osmolarity concentrations are low, cellular levels of phosphorylated transcriptional regulatory protein *ompR* are low, resulting expression of the outer membrane porin *ompF* and repression of the outer membrane porin *ompC*. Conversely, at higher osmolarity concentrations, cellular levels of phosphorylated *ompR* are high resulting in *ompC* is expression and *ompF* is repression. The biological significance of this is hypothesised to be that the larger size of the *ompF* protein results in solute intake under low osmolarity conditions, whereas the smaller porin size in *ompC* results in expulsion of smaller ions, molecules and toxins under high osmolarity conditions (Yoshida *et al*., 2006).

In this study, variants were seen in outer membrane protein transport *phnV* and *clcA.* Furthermore, GO enrichment analysis highlighted the importance of both "integral component of membrane" and "transport activity" to preservative exposed treated Bcc strains. Although little is known about the biological function and mechanism of action, *phnV* is seen to be involved in host infection and persistence in *Pseudomonas aeruginosa* and *Salmonella* serovar Typhimurium (Kim *et al*., 2002). The increased induction of *clcA* as part of host infection and persistence has been demonstrated with various enteric bacteria such as *Salmonella typhimurium*, *E.coli* and *Vibrio cholerae* (Cakar *et al*., 2018; Basilio *et al*., 2014; Iyer *et al*., 2002). Therefore, these outer membrane proteins may also act to increase cellular survival under toxic conditions.

Damage Response

Although, it is not considered a resistance mechanism damage control and compensation of impaired function is integral to bacterial antimicrobial resistance and environmental survival. In this study, variants were seen in genes concerning repairing/reading damaged DNA (*ada*, *alkA* and *dnaE*), in repairing/removing damaged proteins (*hslO* and *htrA*), in removing toxic by-products formed from

oxidative stress/damage (*gpr, speG, puuA, and puuC*) and in transcriptional activators of oxidative damage response pathways (*rclR* and *soxR*).

In this study two of the four genes induced in alkylated DNA repair have variants in the *B. lata* strain 1299 when exposed to BIT preservative. BIT is an oxidiser and acts on the thiol group of cysteine residues, affecting protein folding and function (Maillard, 2002). Thiol free radicals also form the building blocks of DNA and isothiolinone preservatives are seen to affect DNA synthesis (Collier *et al*. 1990). Thiol groups contain an alkyl group and the involvement of alkylating repair enzymes here could be to repair alkyl group thiol damage in DNA.

In this study two gene affecting variants are seen in reactive oxygen/chloride species (ROS/RCS)sensors/response activators. ROS/RCS responses are known to cause damage to integral cellular components including DNA and proteins. *soxR* controls transcription of *soxS* which is a multi-gene transcription activator for superoxide resistance response (Koo *et al*., 2003). SOS response plays a role in both antibiotic and biocide resistance in many bacterial species (Davin-Regli and Pagès, 2015; Condell *et al*., 2014) In various Bcc strains, SOS responses to oxidation can be attenuated using the antioxidant molecule N-acetylcysteine (NAC) and cause growth retardation and biofilm inhibition (Pollini *et al*., 2018). Bacterial RCS tolerance relies on the activation of an RCS resistance system by *rclR*. Whilst the putative function of these genes is unknown, knock outs of any one of these given genes dramatically increased RCS sensitivity (Parker *et al*., 2013). Although, little is known about RCS pathways, and RCS resistance in *Burkholderia* species, RCS induced perioxidases are seen in pathogenic *Burkholderia* species such as *B. cenocepacia* J2315 and *B. pseudomallei (*Peeters *et al*., 2010). It is hypothesised that the involvement of ROS/RCS responses assist in the protection of oxidative DNA damage when Bcc strains are exposed to biocidal preservatives.

Biofilm

In this study, variants were seen in genes affecting signalling of biofilm formation (*gmr*, *mcbR* and *cqsA*), components to assist biosynthesis (*lutP*) and adhesion (*sadA*). The ability of *Burkholderia* species to form biofilms has been researched significantly due to its clinical impact. CF patients infected with Bcc biofilms often

exhibit chronic infection which is extremely difficult to treat due to increased antibiotic resistance and results in a poorer prognosis (George *et al*., 2009). The mechanisms of increased bacterial biofilm antimicrobial resistance result from limited diffusion of the biocide into the biofilm structure, EPS enzymatic breakdown of antimicrobial compounds, increased stress response from inner aerobically and nutritionally deprived cells, decreased antimicrobial activity on metabolically limited cells and the rise of persister sub populations (Maillard, 2018). Phenotypic increases in both biocide and antibiotic resistance are seen for *Burkholderia* species grown in biofilm compared to planktonic bacteria (Rose, 2009). QS signalling is known to be important to biofilm formation. Cyclic di-GMP signalling is integral to pathogenic processes of gram-negative bacteria such as *E. coli* and *Salmonella* spp., with a correlation between cyclic-di-GMP levels, virulence factors (e.g. biofilm synthesis and motility) and clinical outcomes (severe and chronic infections) (Barnhart and Chapman, 2006). Studies on tobramycin susceptibility when QS inhibitors were added found planktonic susceptibilities remained while sessile Bcc strains and *P. aeruginosa* strains displayed increased sensitivity (Brackman *et al*., 2011).

Signalling

Burkholderia species utilise quorum-sensing (QS) circuits for the regulation of survival responses and virulence traits including swarming motility and biofilm synthesis (Eberl, 2006; Venturi *et al*., 2004). Despite the importance, only two shared variants were observed in a QS related homologous genes (*cqsA and mcbR*). Furthermore, cellular signalling was not represented in the GO enrichment analysis. QS expression under stress conditions causes cellular damage through increased ROS, increased susceptibility to antibiotics and hydrogen peroxide synthesis which could account for its lack of involvement in resistance mechanisms of preservative stressed Bcc strains (Häußler *et al*., 2003; D'Argenio *et al*., 2002). This is further supported by a recent study investigating differential gene expression of a Unilever industrial contaminant strain *P. aeruginosa* on exposure to the preservatives BIT, phenoxyethanol and laundry detergent with found quinolone signalling was strongly and repeatedly downregulated in all three conditions (Green *et al*., 2018).

Metabolism

In this study, over half of the shared variants (46/87) and of the GO enrichment analysis (13/21) were directly or indirectly associated with bacterial metabolism. Intrinsic resistance requires a large number of bacterial genes, estimated of up to 3% of the bacterial genome and of genes not just those involved in resistance but also metabolism. For example, mutations in proteins involved in bacterial metabolism decreased bacterial susceptibility to antibiotics (Fajardo *et al*., 2008). Analysis of intrinsic resistance of bacterial pathogens or the intrinsic resistome have demonstrated that a number of genes involved are involved in general bacterial physiology such as metabolism of amino acids, fatty acids and nucleotides and in electron transport chain processes (Zampieri *et al*., 2017). As such potential development of novel antibiotic treatment strategies involve metabolic priming of bacteria pre-antibiotic exposure, through the application of metabolites to increase antimicrobial effect (Vestergaard *et al*., 2017).

Of the metabolism genes, only ones involved in phenylacetate degradation have previously been associated with Bcc antimicrobial resistance (Sass *et al*., 2011). The phenylacetate degradation biochemical pathway is known to involved in *Burkholderia* stress resistance to the host environment including, in nematodes (Law *et al*., 2008), plant rhizospheres (Patrauchan *et al*., 2011) and CF sputum (Yoder-Himes *et al*., 2009). Variation was observed in the phenylacetate degradation complex *paaABCDE* and was associated with stable adaptive preservative resistance. However, an enzyme separate to but also involved in phenylacetate degradation called *paaF* is seen in two other adaptive resistance experiments: H/F adaptive resistance experiment with *B. lata* strain 1299 and benzyl alcohol (no increase in preservative resistance) and H/F adaptive resistance experiment with *B. lata* strain 1299 and phenoxyethanol preservative (transient adaptive preservative resistance) (Appendix 3). Therefore, the involvement of this metabolic pathway is not exclusive to stable preservative resistance but also in response to preservative agent susceptibility and preservative selective pressure.

Despite the mode of action for antibiotics rarely targeting metabolism, antibiotic resistance traits are closely correlated with metabolic processes such as glycolysis, amino acid biosynthesis, respiration and energy metabolism (Zampieri *et al*., 2017).

In the GO enrichment "ATP binding" was significantly enriched. The role of ATP in gram-negative bacterial resistance systems has been studied greatly in relation to the superfamily of drug exporters the ATP-binding cassette family of proteins. This has been studied in most detail with clinically relevant pathogenic organisms such as *E. coli* whereby macrolide antibiotic resistance is mediated through the ABC transporter *MacAB-TolC* (Li *et al*., 2015). In this study variants were seen in three energy dependent transporters *srpABC* and *ttgABC* which are involved solvent and antibiotic efflux and *phnV* involved in metabolite transport. Although, it is unclear by what mechanism, ATP binding plays a role in preservative resistance of Bcc strains.

In this study there were several variants associated with carbon metabolism, including in indirect metabolite transfer for carbon metabolism (*cmpR*, *lutP* and *phnV*), breakdown of metabolites into carbon sources (*puuA* and *puuC*) and in the breakdown of carbon metabolism by-products (*grp*). There have been many studies linking carbon metabolic regulation of antibiotic resistance elements in *Pseudomonas* species (Corona and Martínez, 2013; Morales *et al*., 2004; O'Toole *et al*., 2000). *Crc* (Catabolite Repression Control) is a global control signal, modulating carbon source utilisation in *Psuedomonas* species is also seen to be involved transcriptional control of several virulence factors and antibiotic resistance strategies such as type III secretion systems, motility, biofilm production, porins and transporters (as reviewed in (Martínez and Rojo, 2011). *Crc* is also seen to control various catabolic pathways for the assimilation of aromatic hydrocarbons and amino acids as alternative carbon sources (Moreno *et al*., 2009). Furthermore in *P. aeruginosa*, the *CbrAB* complex which controls both carbon and nitrogen utilisation and antibiotic susceptibility (Yeung *et al*., 2011). Therefore, the presence of variants in Bcc strains exposed to preservatives could demonstrate the involvement of carbon metabolism in the adaptive resistance response of *Burkholderia* species to antimicrobial agents.

In the GO enrichment "Glutamine family amino acid metabolic process" was significantly enriched. Glutamate, an amino acid belonging to the glutamine family of amino acids are seen to act as osmoprotectans in *Burkholderia* species (Behrends *et al*., 2011). Bacteria are also able to utilise different carbon and nitrogen sources

through the metabolism of glutamine family amino acids. In various pathogenic gram-negative species including *P. aeruginosa* and *S. enterica* the virulence trait swarming motility is induced when the glutamine family of amino acids are metabolised as a cellular nitrogen source under limited nutrient conditions (Köhler *et al*., 2000). Swarming cells have increased levels of antimicrobial resistance to antibiotics, heavy metals and biocides when compared to their non-swarming counterparts. This increased resistance could not be attributed to traditional mechanisms such as increased efflux activity. Serval hypothesis have been offered for this phenomenon including changes to the cellular envelope architecture, decreased permeability and increased social signalling alike in biofilms (Lai *et al*., 2009).

5.6 Conclusion

The expression of preservative resistance genes largely could not be associated to preservative or strains and was shared across experimental conditions. Variants associated with stable preservative resistance involved dormancy, signalling, degradation of toxins and metabolism.

For preservative exposed *Burkholderia* strains method and preservative effects were identified. Low and Slow (L/S) adaptive resistance methodology was associated with long term stress resistance strategies while High and Fast (H/F) adaptive resistance methodology was associated with short term damage resistance. For the oxidising agent BIT variants were associated with oxidation damage response whereas for the cell membrane acting benzyl alcohol variants were associated with cell membrane components, and a large proportion of these caused significant damage to protein function.

Genomic analysis of variant function in preservative exposed *Burkholderia* strains identified various known biocide resistance mechanisms including enzymatic degradation, cellular membrane permeability, active efflux, and biofilm synthesis. Furthermore, a significant proportion of variants were observed in mechanisms less associated with biocide resistance such as physical stress resistance, damage response mechanisms, global cellular signalling and metabolism.

Chapter 6: General Conclusions

The objectives of this project were threefold:

To create molecular detection methodologies to enable rapid, efficient and accurate detection and identification of bacterial manufacturing process contaminants from consumer products (Chapter 3: Molecular Detection of Bcc Strains from Personal Care Products).
To determine the preservative susceptibility and adaptive resistance potential of *Burkholderia* challenged with conventional synthetic preservatives and novel natural preservation strategies (Chapter 4: Adaptive Bacterial Resistance to Preservatives).
To investigate the molecular mechanisms driving adaptive preservative resistance (Chapter 5: Genomic analysis of preservative adaptive resistance in *Burkholderia* populations).

The following discussion elaborates on the specific aims as outlined in Section 1.5 Project Aims, key achievements for each chapter, limitations of the work, and future projects which could be investigated following from the research results.

Aim 1: To investigate live/dead staining techniques and RNA abundance in the assessment of contamination viability.

Currently traditional microbiological culture techniques are used to determine microbial contamination and preservative failure. These methods are slow, prone to error and inefficient at identifying certain metabolically static organisms (viable but not culturable) (Denyer, 2006). Molecular technologies could be adopted into quality control procedures, to streamline the supply chain, reduce costs and increase efficiency. Despite this, various hurdles are seen in the application of molecular methods to routine microbiological quality control examination (Orth *et al*., 2006). Whilst molecular methods are sensitive, they are not discriminative for viable and non-viable micro-organisms.

Traditional molecular detection methods in research have focused on RNA as the target for nucleic acid detection protocols (Tan and Yiap, 2009). rRNA indicates cell viability and offers a better likelihood of low-level detection due to an increased relative abundance in bacteria compared to DNA (Cenciarini-Borde *et al*., 2009). In this study, evaluation of RNA as a target for isolation found RNA is complex to isolate due to its poor stability and requires careful processing including a long treatment with an RNA stabilisation agent and conversion to cDNA. Whilst cellular abundance of RNA is higher in normal bacterial cells, stressed bacteria downregulate transcriptional activity resulting in a reduction in RNA. Comparably when RNA or DNA was simultaneously extracted from artificially contaminated personal care products using a broad range detection methodology, amplification of RNA was comparative to DNA. Therefore, whilst research methods suggest RNA is a preferred target for detection, the physiology of stressed bacteria that are present as industrial contaminants and the need for a quick protocol suggests that DNA is preferable for use in the manufacturing industry.

Viability staining techniques incorporate a pre-PCR treatment with dye which can selectively bind to the DNA of dead cells to inhibit their amplification in PCR (Zeng *et al*., 2016; Elizaquível *et al*., 2014). Ethidium monoazide/PCR methods (EMA PCR) were evaluated for their ability to provide an estimate of live and potentially pathogenic bacteria from cultures and artificially contaminated personal care products. In this study visualisation of dead cell DNA using the EMA PCR approach did not significantly differ from PCR approaches without EMA. Due to time constrains a full comprehensive validation of EMA PCR could not be undertaken. The utility of EMA PCR is complicated due to the availability of various stains and the time-consuming process of methodological validation regarding stain concentration and incubation times. Even with the availability of commercial staining kits, protocol amendments are usually required (Davey, 2011). Further work could validate the utility of viability stains such as EMA and propidum iodide (PI) and/or commercial staining kits to multi-species of industrial contaminants for use in molecular identification methods.

Aim 2: To develop a broad range, quantitative, rapid molecular method to detect and identify low level bacterial contamination (less than or equal to 10 CFU/g) in a variety of non-sterile home and personal care consumer products.

The advent of the genomic age has driven new levels of microbial discovery and taxonomic identification (Taboada *et al*., 2017). Until recently, advancements in molecular technologies have only been utilised by research laboratories due to their requirement of costly sophisticated equipment and expert operators. However, protocols have become cheaper, easier to operate and more accessible.

Despite various attempts at optimisation of sample treatment, organism capture and lysis, target enrichment, target amplification and target detection the best detection sensitivity achieved was 10^6 CFU/ml, much greater than the $<10^3$ CFU/ml target.

Home and personal care products are often viscous matrices comprised of detergents, surfactants, and antimicrobial substances. Extracting low levels of physiologically stressed bacteria from this difficult matrix posed many difficulties. The biggest issue facing the applicability of molecular methods to QC microbial detection and identification is the requirement of dilution of viscous product matrices which reduces detection sensitivity. However, micro technology systems such as microfluidic devices can be used with single cell concentrations (Wu *et al*., 2018; Murphy *et al*., 2017). These devices can be made cheaply using soft lithography, enable accurate microbial detection, produce rapid results (1-day turnaround), are high throughput and fully automated. Microfluidic chips can be coupled with on-chip identification techniques such as Polymerase Chain Reaction (PCR) and Loop Mediated Isothermal Amplification (LAMP) to enable post detection microbial identification (Mauk *et al*., 2018). The capabilities of these devices are only just beginning to be explored in molecular diagnostics and molecular identification (Zhang *et al*., 2018). For QC microbial detection, microfluidic chips could be integrated into production pipelines, allowing real time product analysis. At present, molecular methods are designed for small sample throughput are not robust enough for the everyday QC demands of the factory environment which requires rapid high-volume sample analysis at a low cost per sample and with minimal operator skill and labour. However, future development of microfluidic based instrumentation may

offer a sensitive, low-cost, quick, reliable and simple to operate microbial detection and identification which should be explored further.

Aim 3: To develop a rapid narrow range detection and identification protocol for suspected product contamination. To assess the efficacy of the protocol against real world contamination samples from across Unilever home and personal care division.

In the rare occurrence of a contamination event, an agile response from QC and QA departments is required to isolate affected product batches and determine the organism of cause. Quick discovery is essential to mitigate economic losses that arise from warehouse costs for storage for suspect products whilst additional tests are performed, production line/plant closure and product recall (Nemati *et al*., 2016). Conventionally routine screening of products is performed using culture-based methods. Once an out of specification result is returned, additional culture-based methods are employed for identification, usually through biochemical tests and species-specific agar. However, along with being slow these methods are poor at correctly identifying closely related bacteria and ineffective against viable but not culturable bacterial species (Denise Donoghue, personal communication). Molecular biological methods could be of great utility in providing greater resolution to out of specification results, and provide both detection and identification information accurately, quickly and efficiently. To demonstrate utility in the factory QC environment, assays would need to detect out specification levels contamination (10^3 CFU/ml) in a variety of sample matrices, within a "7hr shift" timeframe, and provide a distinction between the two most common bacterial spoilage organisms: *Burkholderia* species and *Pseudomonas*.

In this study the direct use of crude lysates and boil-prep treatments with nested PCR techniques were able to provide detection sensitivity of $>10^3$ CFU/ml within a four-hour period. Application of this lysis buffer boil prep with nested PCR methodology on real world archived contamination samples was highly effective, and was able to identify contamination in all samples, even without viable counts. However, the utility of this method is limited due to the lack of sensitivity and specificity with 16s rRNA *Burkholderia* and *Pseudomonas* primers which could not provide an accurate

identification of contaminating organisms. In comparison to current out of specification factory detection protocols, this method offers a feasible alternative to current QC detection and identification methods. Current scientific literature surrounding molecular methods of detection in routine testing laboratories (clinical and industrial) often require laborious and costly pre-processing treatments such as nucleic acid extraction methods (Buchan and Ledeboer, 2014; Ceuppens *et al*., 2014). This method requires limited skill and processing, making it user friendly, cost efficient and timesaving.

The utility of this method could be greatly improved with validation of *Burkholderia* and *Pseudomonas* specific primers. The ability to detect and accurately identify Bcc strains via molecular methods has been difficult to achieve due to an estimated 97% sequence homology between members of the Bcc (Ragupathi and Veeraraghavan, 2019; Coenye *et al*., 2001). Further work could address the creation of discriminatory *Burkholderia* and Psuedomonas specific PCR primers through recA gene-based identification (Kong *et al*., 2012) or MLST (Gautam *et al*., 2016). . This could also be extended in multiplex / nested PCR methods to enable simultaneous detection.

Aim 4: To determine the preservative susceptibility of a large collection of Bcc clinical and industrial contaminant strains against synthetic and natural preservative agents using minimum inhibitory concentration methodologies.

Preservatives are antimicrobial compounds added to raw materials and manufactured products to ensure microbiological integrity is maintained during manufacture, storage and consumer use. Due to their presence in daily personal care and house-hold products, preservatives are the most widely circulated antimicrobial (SCENIHR, 2009). Despite their extensive use little is understood about preservative resistance. While preservatives are classified as a subset of biocidal compounds, biocide resistance research has centred around antiseptics and disinfectants as it pertains to clinical outcomes (Maillard *et al*., 2013). However, preservation is rather different to antiseptics and disinfectants, notably that in-use concentrations of preservatives are much lower than antiseptics and disinfectants due to consumer toxicity concerns (Gilbert and McBain, 2003). Currently *in-situ* resistance has been

identified for commonly used preservatives such as: Benzalkonium chloride (Abdelaziz *et al*., 2019; Ahn *et al*., 2016; Guo *et al*., 2013; Tandukar *et al*., 2013; Nagai *et al*., 2003; Joynson *et al*., 2002; Kurihara *et al*., 1993), isothiazolinones (Green *et al*., 2018; Rushton *et al*., 2013; Winder *et al*., 2000; Sondossi *et al*., 1999; Chapman *et al*., 1998), DMDMH (Rushton *et al*., 2013; Chapman et al., 1998), cetylpyridinium chloride (Latimer *et al*., 2015; Rose, 2009) and phenoxyethanol (Green *et al*., 2018; Rushton *et al*., 2013; Malek and Badran, 2010). Presently resistance to all known preservatives has been reported (SCENIHR, 2009; Chapman, 1998; Chapman *et al*., 1998; Flores *et al*., 1997).

Historically preservatives used in the manufacturing industry are synthetic/inorganic compounds. In recent years regulatory changes and a shift in consumer demand for more natural products has led to the industry exploring natural preservation options (Reisch, 2016, 2018). Exploring alternative preservative models can potentially circumvent preservative resistance since sub-inhibitory concentrations of common preservatives are known to promote preservative-adaptive resistance mechanisms in bacterial species (Gilbert and McBain, 2003).

Typically, natural preservatives are antimicrobial compounds of botanical origin but chemically synthesised (Ibarra and Johnson, 2008). It is assumed that nature identical preservatives have weaker antimicrobial properties than their synthetic counterparts, require higher in-use concentrations, lack efficacy against bacteria and are variable in activity (Browne *et al*., 2012). Empirical evidence regarding the comparative antimicrobial efficacy of synthetic and nature-identical preservatives is lacking. In this study the utility of nature-identical preservatives to inhibit environmental Bcc contaminant strains was investigated. All *Burkholderia* strains were effectively inhibited by the nature identical preservatives benzyl alcohol, phenoxyethanol, benzoic acid and salicylic acid at concentrations that did not exceed the maximum amounts permitted by the EU cosmetics directive EEC/76/768/EEC. Benzoic acid and benzyl alcohol preservatives required high in use concentrations to demonstrate efficacy, but this was comparable or lower to the synthetic preservative DMDMH and marginally higher than methyl paraben (<2-fold difference). Salicylic acid and phenoxyethanol demonstrated in use concentrations lower than the synthetic preservatives DMDMH and methyl paraben. This is also supported by a recent paper which compared synthetic and nature-identical preservative

antimicrobial efficacy within an emulsion product against four challenge organisms (*C. albicans, S. aureus, P.aeruginosa, A. Brasiliensis*) and found that nature identical preservatives had better antimicrobial activity than some synthetic agents (Glavač and Lunder, 2018). This demonstrates the potential utility of nature identical preservatives in product preservation. Future product preservation strategies should explore the use of nature identical preservatives, especially in substitution of synthetic preservatives such as DMDMH and methyl paraben as nature identical preservatives exhibit antimicrobial efficacy at similar or lower concentrations than these synthetic compounds.

There is also a concern that nature identical preservatives are variable in activity (Browne *et al*., 2012). This study found the that organic acids had poor stability leading to variable quality in activity. However, nature identical preservatives based on alcohols offer a stable and effective alternative. For example, the chemical properties of phenoxyethanol are compatible with various product formulations, with a stable activity at a wide pH range (3 - 8.5) and an easily soluble liquid form.

In this study nature identical preservatives had higher in use concentrations than isothiazolinone preservatives. The synthetic isothiazolinone preservatives had the lowest in use concentration and could be used in concentrations 100-fold lower than nature identical preservatives. However, use of isothiazolinone preservatives is heavily restricted in the EU due to sensitisation issues (Gameiro *et al*., 2014). BIT is only regulated for use in industrial products such as paints, emulsions and adhesives and is not permitted for use in household or personal care products. On the 22nd July 2016 the European Commission banned methylisothiazolinone in leave-on products, and from 6 July 2017 amounts of methylisothiazolinone in rinse-off products was significantly reduced from 0.01% to 0.0015% (European Commission, 2016, 2017) .

In product preservation, manufacturers have to navigate not only regulatory confines but also public opinion. Although the nature identical preservatives used in this study had greater in-use concentrations (and would therefore be more expensive as preservative agents), consumers are looking for manufacturers to provide safer and greener alternatives, necessitating the investigation of nature-identical preservative agents.

Despite the promising efficacy of nature identical preservatives, there are various concerns of how the results obtained from *in vitro* preservative susceptibility relates

to in vivo formulation preservation. The majority of biocide and preservative susceptibility testing is undertaken with the biocide in isolation, and not in its intended format, combined with product formulations (SCENIHR, 2009). Formulations may affect the efficacy of the biocide, for example a 0.5% benzethonium chloride was less effective in a shampoo formulation when challenged with *Burkholderia* and *Salmonella* strains than when tested alone (Knapp, 2014). Pharmacopeia chapters USP <51>, EP 5.1.3 and JP 19 dictate requirements of in-formulation antimicrobial efficacy tests called Preservation Efficacy Tests (PET). Future work could focus on comparative examination of the preservation efficacy of synthetic and nature identical preservatives when incorporated into home and personal care formulations.

Understanding the interaction between bacteria and different preservatives is important in order to combat preservative resistance. Pan preservative resistance was not demonstrated, nor could a correlation be made for resistance to classes of preservative, or resistance by species groups. For example, *B. lata* strain 1299 exhibited intrinsic resistance to the synthetic preservative BIT 10 times greater than any other *Burkholderia* strain, however preservative susceptibility to other agents, including the related M-CMIT did not exceed values seen in other BCC strains. This follows current research where *Burkholderia* biocide and preservative resistance is dependent on individual strain and agent factors (Rushton *et al*., 2013).

Preservative susceptibility tests were performed using a *Burkholderia* stain panel encompassing 40 species isolated from environmental-industrial niches and 7 from clinical locations. Other studies have demonstrated a significantly lower preservative tsusceptibility to DMDMH preservative for environment-industrial *Burkholderia* isolates compared to clinical (Thomas, 2011; Rose, 2009). Small sample size made it difficult to compare the preservation susceptibility of clinical *Burkholderia* isolates to environmental-industrial isolates. However, average MIC's for select preservatives were seen to be greater in environmental industrial isolates. Future work incorporating a larger BCC strain panel would enable examination of differences between clinical and environmental-industrial BCC isolates in preservative resistance.

In this study preservative susceptibility testing was performed using agar and both dilution methodologies challenged with media grown *Burkholderia* strains. In the industrial manufacturing environment, bacteria exist most commonly in biofilms (Satpathy *et al*., 2016). Biofilms are able to adhere to internal components of complex production equipment and water systems which confers greater protection from physical stressors e.g. temperature or impact from water/materials and chemical cleaning products (Dufour *et al*., 2010). Comparisons of the susceptibilities of biofilm grown and planktonic *Burkholderia* cells demonstrate that biofilm have a greater natural resistance to antimicrobials (Murphy and Caraher, 2015; Murphy *et al*., 2013; Coenye *et al*., 2011; Rose, 2009; Caraher *et al*., 2007; sai *et al*., 1998). At present, the susceptibility of *Burkholderia* biofilms to synthetic preservatives has been limited to chlorohexidine (Coenye *et al*., 2011) and the effect of nature identical preservatives has not been studied. Therefore, repeating preservative susceptibilities with biofilm grown *Burkholderia* strains would be worthy of exploration.

Antimicrobial susceptibility testing procedures rely on traditional culture-based microbiology approaches which are time consuming and inaccurate. In clinical antimicrobial susceptibility testing, automated optical detection instruments have addressed the growing need for standardised and sensitive antimicrobial sensitivity testing (Reller *et al*., 2009). Due to poor solubility and opacity of biocidal compounds, these imaging-based systems could not be utilised. Replication independent bacteria detecting technologies which utilise DNA probes that hybridise to molecular markers have been explored as antimicrobial susceptibility testing workflows, including qPCR approaches (Luo *et al*., 2018; Hemarajata *et al*., 2016). The current use of qPCR approaches with *Burkholderia cepacia* complex organisms is hindered by poor performance of available *Burkholderia* qPCR primers and limited genomic information for primer design. The current development of Smarticles (Roche, USA) which uses probe carrying bacteriophages to target bacterial DNA and omit a measurable luciferase signal for drug resistant bacterial cells enables rapid and accurate antimicrobial susceptibility testing. While not currently available, initial validation studies have found antimicrobial susceptibility testing and therapy initiation time was cut from 81 hours to 21 hours, hospital stays were reduced 34% and mortality more than 50% (Anon, 2015). This could enable

manufacturers to test a multitude of alternative preservation systems against a robust and diverse panel of organisms quickly and reliably. Once commercially available, the utility of this method to detection and preservation susceptibility testing in industry should be seriously considered. Automated instruments which detect living bacterial cells through dyes and biochemical signals as a proxy for bacterial presence and abundance such as ATP bioluminescence meters and flow cytometry machines have also been employed for rapid antimicrobial susceptibility testing (Steinberger-Levy *et al*., 2016; Broeren *et al*., 2013; Nuding, 2013). While these are faster and more cost effective than traditional antimicrobial susceptibility culture techniques, they require time consuming pre-enrichment/lysis steps, are non-specific to any micro-organism, lack sensitivity at low antimicrobial concentrations and are not universally compatible with all micro-organisms (Orth *et al*., 2006). Nonetheless, their utility in preservative susceptibility testing could be explored.

Aim 5: To investigate the effect of strain/species, preservative type and adaptive resistance method on the potential generation of resistant populations by exposure to increasing preservative concentrations.

Unlike biocides and disinfectants which are used at bactericidal concentrations, preservative agents are often used at their minimum efficacy to prevent interference to the formulation or negative effects to the user (i.e. sensitisation effects) (Wales and Davies, 2015). Low preservative product concentrations make them vulnerable to dilution through degradation and formulation interaction (Hugo, 1991). Furthermore, in the industrial environment there may be a spectrum of sub-inhibitory antimicrobial concentrations which occur through improper sanitation of machinery and production facility (Maillard, 2002). Exposure of bacteria to sub-inhibitory concentrations of preservatives can increase tolerance mechanisms thereby inducing adaptive resistance (Fernández *et al*., 2011). Adaptive resistance is extremely important to the initial response to an antimicrobial and may even affect long term resistance profiles. There is a proposed link between the exposure length and dose of antimicrobial agent and the stability of adaptive resistance phenotypes (Motta and Aldana, 2016). At present the potential for *Burkholderia* species to adapt to current and novel preservation strategies has not been extensively explored.

The potential induction of preservative adaptive resistance was not associated by species, as resistance varied between strains within the *B. cenocepacia* species. Preservative tsusceptibility did not predict adaptive resistance potential. *B. lata* strain 1299 displayed a ten times greater innate resistance to BIT however the stability or level adaptive resistance gained by this organism to other preservatives did not differ from other BCC strains tested. This is corroborated by recent micro-evolution experiments which found sensitive *E. coli* mutants were able to develop greater resistance over time than mutants that demonstrated high initial antibiotic resistance (Baym *et al*., 2016).

For both phenoxyethanol and BIT preservatives exposure to sub-inhibitory preservative concentrations resulted in stable adaptive preservative resistance. Despite increases preservative resistance did not breach maximum permitted in use concentrations. However, adaptive resistance and the increase of basal levels of antimicrobial resistance is attributed to conference of high-level antimicrobial resistance (Motta and Aldana, 2016). Stable adaptive resistance to the nature identical preservative benzyl alcohol could not be promoted in BCC strains. This is important to home and personal care manufactures' who must construct high efficacy eco-friendly preservation systems which do not pose a risk of future contamination from bacterial adaptive resistance.

In this study, two methodologies were designed to induce different types of preservative adaptive resistance through manipulation of timeframe and antimicrobial gradient. In contradiction to current theory, stable adaptive preservative resistance to phenoxyethanol and BIT was promoted by short exposure and high concentrations of preservative agent along with slowing increasing gradients over extended periods. Prolonged exposure to slowly increasing gradients of phenoxyethanol and BIT preservatives resulted in elevated preservative resistance levels, when compared to higher concentration gradients and short exposure periods. For prolonged exposure to slowly increasing sub-inhibitory gradients the trajectory of preservative resistance increased steadily. Whilst for higher concentration gradients and short exposure periods resistance was variable passage to passage. This demonstrates that stable adaptive resistance is not limited to slow and steady mechanisms. However, the potential of adaptive resistance was greater for slowly

increasing sub-inhibitory gradients than for higher concentration gradients and short exposure periods. Further examination of the timescales required for induction of high-level adaptive resistance could be performed over an extended period. This demonstrates the importance of sub-inhibitory exposure on the development of high-level adaptive resistance to preservative agents.

Values obtained from adaptive laboratory experiments indicate the potential generation of bacterial adaptive resistance to antimicrobial agents. However, these are often performed with bacterial cells grown in a homogenous culture within an antimicrobial spiked nutrient rich broth which is not reflective of true environmental conditions. Mathematical modelling showed that antimicrobial environments contain gradients which enable bacteria to persist in low concentration "sanctuaries" (Hermsen *et al*., 2012). From this protective special zone bacteria can slowly adapt and branch out into other areas of higher concentration. This concept of spatial microbial evolution has been demonstrated eloquently through time-lapse recordings of *E. coli* on a large petri-dish containing gradients of antibiotics (Baym *et al*., 2016). Biofilms provide another example of how protected "sanctuaries", found within the innermost biofilm structure enable growth into higher concentration gradients (Sánchez-Romero and Casadesús, 2014; Stewart *et al*., 2010; Szomolay *et al*., 2005; Mah *et al*., 2003). Bacteria in the natural environment, including as industrial contaminants are commonly found to be growing in biofilm structures. However, little is known about the induction of preservative adaptive resistance and the mechanisms involved in preservative resistance of bacterial biofilms. As biofilms exist in a very different physiological and metabolic state to planktonic cells, it is likely that the mechanisms of resistance would be distinctive and warrants further investigation. Future studies of the adaption potential of *Burkholderia* to preservatives could employ spatial microbial evolution methods or biofilm grown cells to enhance the understanding of potential induction of preservative adaptive resistance in a more realistic environment.

Aim 6: To investigate the molecular basis for antimicrobial preservative resistance through whole genome sequencing, Single Nucleotide Polymorphism analysis and GO enrichment.

BCC strains are intrinsically resistant to antimicrobials, as demonstrated by their frequent isolation as industrial contaminants and as multi drug resistant opportunist pathogens (Coenye and Mahenthiralingam, 2014). Developments in molecular research technologies and the explosion of the omics field have given greater understanding into the mechanisms involved in the emergence and dissemination of antibiotic resistance. Despite these advances, current understanding behind the mechanisms of preservative resistance are still relatively unknown. Unlike antibiotics, preservative affect multiple targets and resistance mechanisms are thought to be multifactorial and complex (Wales and Davies, 2015).

Variants were called, filtered and annotated for functional effects in the 22 *Burkholderia* genomes. Experimental variants were then filtered against controls of *B. lata* strain 1299 and *B. cenocepacia* strains 1291, 1292 and 1318, grown without preservative. Four strains were omitted for poor mapping quality and large number of variants. For the available data on 18 experiments a total of 1465 variants were identified. There was a statistically significant correlation observed for experimental set up and the number of variants in sequenced isolates. The low gradient and slow timescale (L/S) adaptive resistance methodology demonstrated larger number of genomic variants than sequenced isolates from high gradient and fast timescale (H/F) methodologies. There was no correlation for strain, species or preservative type on the number of variants observed in sequenced isolates.

In this study mutated genes were cross referenced across experiments and strains, and if present in two or more experiments it was considered to be selected for (Jahn *et al*., 2017; Sandberg *et al*., 2014; Lieberman *et al*., 2011; Yang *et al*., 2011). Various putative candidate genes were identified to be involved in preservative induced adaptive resistance of BCC strains. However, candidate genes would require further experimental investigation using directed mutagenesis and complementation studies to demonstrate their functional contribution to resistance or survival in toxic environments, if any.

Variants were identified in 13 genes from sequenced *Burkholderia* isolates that displayed stable preservative resistant phenotypes. These variants were indicated in processes in-directly involved in antimicrobial resistance. The majority of the variants were associated with metabolism (7/13) and stress resistance (4/13). The remaining two were observed with signalling and enzymatic detoxification.

Wider functional analysis was undertaken of 74 shared genetic variants in sequenced isolates under selective pressure. 43 variants were associated with metabolic/cellular activities as of yet un-associated with resistance. The majority of the remaining 31 concerned genes associated with indirect resistance mechanisms. Nine genes were associated with stress resistance mechanisms and seven genes were involved in the degradation of toxins. The remaining genes correlated with classical resistance mechanisms such as efflux pump systems, biofilm synthesis and cellular membrane. GO enrichment analysis confirmed the significant involvement of abstract resistance determinants such as transport, xenobiotic degradation and metabolism in isolates under preservative selective pressure. This is in keeping with the current understanding of adaptive and intrinsic antimicrobial resistance. Current classical resistance determinants do not account fully for the expression of resistance phenotype and activation of indirect resistance determinants may contribute greatly (Cohen *et al*., 2013). Fundamentally BCC bacteria are able to deal with physiologically extreme conditions such as nutrient starvation and environmental toxicity which is essential to survival and proliferation in preserved raw materials and finished products.

The presence of variants in preservative exposed BCC strains was affected by various factors including exposure method, strain and agent. The intrinsically BIT resistant *B. lata* strain 1299 demonstrated 4 unique genes variants which were associated with damage resistance. BIT preservative is known to be a potent oxidiser and therefore induction of damage response genes would afford significant protection (Halla *et al*., 2018). Benzyl alcohol preservative was associated with gene variants in efflux pump constituents and was also correlated with high and moderate protein affecting variants in cellular membrane components. Benzyl alcohol is known to act on prokaryotic cell membranes even at low concentrations (Yano *et al*., 2016). Furthermore, phenotypic adaptive resistance to benzyl alcohol could not be

induced. The observed damage to integral outer membrane components and the inability to induce preservative adaptive resistance demonstrates the utility of this nature identical preservative to the manufacturing industry.

The putative function of gene variants was observed to correlate with adaptive resistance method. The majority of gene variants shared with high concentration and fast time scale methodology and low concentration slow timescale methodologies related to genes involved in broad resistance to antimicrobial agents such as efflux pumps and damage responses. Gene variants seen in low concentration and slow timescale experiments were mainly concerned with stress resistance which illustrates the importance of long term cellular compensatory mechanism to preservative resistance. The two genes observed with high concentration and fast time scale experiments are both seen to contribute to the degradation of toxic substances which indicates short term detoxification processes are important to cellular preservative survival. Current theory suggests that slow and stable mechanisms of adaptive resistance (SSM) occurs through the induction of mutation compensation whilst fast and transient mechanisms of adaptive resistance (FTM) is the product of largely innate factors (Motta and Aldana, 2016). BCC bacteria are highly resistant to antimicrobials and this is caused in part by their natural biological capabilities (Mahenthiralingam and Vandamme, 2005). Exposure to low and sub-lethal concentrations of biocides exerts selective pressure for bacteria with an array of different physiological and genetic adaptions to mitigate damage and enhance survival (Gilbert and McBain, 2003). These mechanisms are diverse and include the induction of efflux pumps to expel toxic substances, the activation of a stress response to restore osmotic and metabolic homeostasis and enhancing enzymatic degradation pathways.

Bacteria when exposed to gradients of antimicrobial substances can exhibit adaptive resistance through gene mutations and/or gene expression changes (Fernández *et al*., 2011). Transient adaptive resistance traits are associated with gene expression changes while stable adaptive resistance traits are associated with the selection of beneficial mutations (Motta *et al*., 2015). In this study GO enrichment indicated that methylation, which is involved in heritability of DNA methylation patterns which is hypothesised to be involved in bacterial adaptive resistance (Motta and Aldana,

2016). The gene expression profiles of preservative induced adaptive resistant *Burkholderia* strains were not examined. Real-time PCR and/or microarray techniques which allow investigation of gene expression changes have been previously used to identify the genomic mechanisms of biocide induced adaptive resistance in various bacterial strains (Lin *et al*., 2017; Curiao *et al*., 2015; Rushton *et al*., 2013; Sass *et al*., 2011; Whitehead *et al*., 2001). Employing real-time PCR and microarray techniques could aid greater understanding of mechanisms which enable *Burkholderia* strains to become more resistant to preservatives and therefore could be utilised in future work.

It is acknowledged that when bacterial populations are exposed to antimicrobials during bacterial adaption experiments, there are a diverse range of responses both inter and intra bacterial populations. This makes it complex to identify common associated traits (Erickson *et al*., 2017). Repeating the adaptive evolution protocol and sequencing of adapted derivatives could assist in identifying mutations systematically under selection when exposed to sub-inhibitory concentrations of preservatives. This approach has been used recently with industrial contaminant *P. aeruginosa* strains which were exposed to preservatives on 5 separate occasions and gene expression profiles of each experiment were corroborated to find commonly upregulated/downregulated genes in response to preservative exposure (Green *et al*., 2018).

Chapter 7: References

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Appendices

Appendix 1: Description and usage instructions for all scripts used in thesis

Table 1: Assessing quality of Illumina Data

Description	Task	Programme	Command
Aligning Illumina files to PacBio references	Compile Bowtie2 reference files	Bowtie2 Version 2.2.5	Bowtie2 -build <reference.fasta> <bowtie_index.bt2> reference.fasta = Polished SMRT PORTAL Assembly Genome bowtie_index.bt2 = Corresponding index for reference genome
	Align with Bowtie2		Bowtie2 -x <bowtie_index.bt2> <forward.fastq.gz> <reverse.fastq.gz> > <output.sam> forward.fastq.gz/ reverse.fastq.gz = Illumina paired read data in zipped fastq files bowtie_index.bt2 = Corresponding index for reference genome

Table 2: Variant calling analysis

Description	Task	Programme	Command
Pre-processing Illumina fastq files for use in GATK	Compile BWA Indexes	BWA Version 0.7.12	bwa index <reference.fasta> reference.fasta = Polished SMRT PORTAL Assembly Genome
	Align with BWA		bwa mem -M -R '@RG\tID:group\tSM:Sample1\tPL:illumina\tLB:lib1\tPU:Unit1'<bwaINDEX.fasta> <forward.fastq.gz> <reverse.fastq.gz> > <aligned_reads.sam> '@RG\tID:group\tSM:Sample1\tPL:illumina\tLB:lib1\tPU:Unit1' = Read Group information for sample forward.fastq.gz/ reverse.fastq.gz = Illumina paired read data in zipped fastq files bwaINDEX.fasta = Pre-compled BWA indexed corresponding to Reference Genome
	Convert SAM to BAM File	Samtools Version 1.3	Samtools views -Sb < aligned_reads.sam> < aligned_reads.bam>
	Mark and Remove Duplicates	Picard Tools Version 2.16.0	java -jar picard.jar MarkDuplicates <aligned_reads.bam> <marked_duplicates.bam> REMOVE_DUPLICATES <marked_dup_metrics.txt>

Table 2: Variant calling analysis (Continued)

Description	Task	Programme	Command
Call haplotypes using GATK	Haplotype Calling	GATK Version 3.7	Java -jar GenomeAnalysisTK037.jar -T HaplotypeCaller -R reference.fasta -I sorted_reads.bam --genotyping_mode DISCOVERY --max_num_PL_values 1771 -stand_call_conf 10 -ploidy 20 -o sample.vcf Notes: --genotyping_mode DISCOVERY : The genotyper will choose the most likely alternate allele Maximum number of PL values to output :Where PL =the likelihood of the genotype (which is determined by the ploidy and the number of alleles) as calculated by normalised phred-scaled likelihoods. As this is a bacterial population with a high ploidy, the maximum number of PL values is high as was adjusted to the highest PL seen (1771). -stand_call_conf : This is the minimum phred-scaled confidence threshold at which variants should be called. This value is intentionally low to enable high sensitivity however the increased chance of false positives means filtering step is recommended after variant calling. -ploidy : Ploidy per sample with the assumption that samples are genetically homogeneous. As this is a bacterial population and we are interested in capturing allelic divergence the sample is treated as a pool of samples and the ploidy is set at the highest possible number 20. Currently GATK does not support a higher ploidy. The allele frequency resolution of this ploidy gives a chance of capture alleles present in 5% of the cellular population.

Table 2: Variant calling analysis (Continued)

Description	Task	Programme	Command
View Variant Information	Output Variant Information to Table	GATK Version 3.7	java -jar GenomeAnalysisTK037.jar -R reference.fasta -T VariantsToTable -V sample.vcf -F CHROM -F POS -F ID -F REF -F ALT -F AF -F QD -F FS -F MQ -F MQRankSum -F ReadPosRankSum -o results.table Notes: CHROM = Chromosome on which the variant occurs POS = Genomic coordinates on which the variant occurs REF = Reference Allele ALT = Variant Allele AF = Allele Frequency QD = QualityByDepth, the phred scale probability an allele exists at random (QUAL field) divided by the unfiltered depth of non-homologus-reference. FS = FisherStrand, the Phred-scaled p-value using Fisher's Exact Test to detect strand bias. If the variation being seen in one direction only then this is indicative of a false positive. MQ = RMSMappingQuality, the Root Mean Square of the mapping quality of the reads across all samples. MQRankSum = MappingQualityRankSumTest, the u-based z-approximation from the Mann-Whitney Rank Sum Test comparing mapping qualities for reads with ref bases and those with the alternate allele. ReadPosRankSum = ReadPosRankSumTest, the u-based z-approximation from the Mann-Whitney Rank Sum Test analysing the distance of the variant from the end of the read, with errors at read ends indicative of sequencing error.
Filtering Variants	Extraction of Raw SNPS	GATK Version 3.7	java -jar GenomeAnalysisTK.jar -T SelectVariants -R reference.fa -V raw_variants.vcf -selectType SNP -o raw_snps.vcf

Table 2: Variant calling analysis (Continued)

Description	Task	Programme	Command
Filtering Variants	Application of SNP Filter	GATK Version 3.7	java -jar GenomeAnalysisTK.jar -T VariantFiltration -R reference.fa -V raw_snps.vcf --filterExpression "QD < (Value of top 10% for that sample) || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0" --filterName "my_snp_filter" – out_mode EMIT_VARIANTS_ONLY -o filtered_snps.vcf Notes: As all annotations are guidelines, a hard filter keeping only the top 10 % of quality (QD) ranked SNPs and Indels was chosen to account for wide differences in quality seen between samples. All other quality recommendations were followed.
	Extraction of Raw Indels	GATK Version 3.7	java -jar GenomeAnalysisTK.jar -T SelectVariants -R reference.fa -V raw_variants.vcf -selectType INDEL -o raw_indels.vcf
	Application of Indel filter	GATK Version 3.7	java -jar GenomeAnalysisTK.jar -T VariantFiltration -R reference.fa -V raw_indels.vcf --filterExpression "QD < (Value of top 10% for that sample) || FS > 200.0 || ReadPosRankSum < -20.0" --filterName "my_indel_filter" – out_mode EMIT_VARIANTS_ONLY-o filtered_indels.vcf Notes: As all annotations are guidelines, a hard filter keeping only the top 10 % of quality (QD) ranked SNPs and Indels was chosen to account for wide differences in quality seen between samples. All other quality recommendations were followed.
	Keeping Variants that Passed VCF Filter		awk '/^#/||$7=="PASS"' 1snps.vcf > passed1snps.vcf
	Creating a New VCF Index	GATK Version 3.7	java -jar ~/bin/GenomeAnalysisTK037.jar -T SelectVariants -R reference.fasta -V 1snps.vcf -selectType SNP -o passed1snps.vcf

Table 2: Variant calling analysis (Continued)

Description	Task	Programme	Command
Cross referencing SNPs to Genome	Annotate Reference genomes	PROKKA Version 1.12	Prokka --outdir Prokka --prefix strain_number reference.fasta --genus *Burkholderia* –species species --strain strain_number –gram neg reference.fasta = Polished SMRT PORTAL Assembly Genome
	Build reference database in snpEff	snpEff Version 4.3	snpEff/data/burkspecies.strain/genes.gff java -jar snpEff.jar -build -gff3 -v burkspecies.strain genes.gff = PROKKA Annotated Genome burkspecies.strain = Species/strain of Reference added in snpEFF directory
	Annotate VCF files	snpEff Version 4.3	java -jar snpEff.jar burkspecies.strain X > Y -csvStats 1snps.stats X = Experimental VCF File Y = Annotated Experimental VCF File
Isolating Genes of Interest	Extract Variant and Gene Name Information From VCF	snpSift Version 4.3	java -jar SnpSift.jar extractFields Y “CHROM” “POS” “AF” “ANN.GENE:” >1snps.txt Y = Annotated Experimental VCF File
	Deleting Duplicates within Experimental Files and Controls	Microsoft Excel Version 16.23	=IF(ISNUMBER(MATCH(X, Y:Y, 0)), “YES”, “NO”) X=Gene of Interest Y:Y=All Genes in Files If X and Y genes match then output is YES. For gene matches within files AF were concatenated. For gene matches in control files, genes were removed from experimental files.

Table 2: GO Enrichment Analysis

Description	Task	Programme	Command
Make topGO read mapping with reference files	Create and edit properties file	Blast2Go Version 1.3.3	blast2go_cli.run -properties cli.prop
	Run blast2go with properties file and references	Blast2Go Version 1.3.3	blast2go_cli_v1.3.3c2/blast2go_cli.run -properties cli.prop -localblast ~/bin/ncbi-blast-2.6.0+/bin/nr.pal -loadfasta fasta.faa -ips <valid email> -protein -exportgeneric ~/b2gWorkspace/saved.txt -savelog ~/b2gWorkspace/saved.log fasta.faa = Prokka Annotated Reference Genome
Editing topGO Experimental Input Files	Add Significance Values to Genes Containing Variants	Microsoft Excel Version 16.23	Notes: Experimental and control data was concatenated to provide a list of genes containing SNPs/Indels with a value of significance. The experimental genes were valued at "0" to show significance while the control genes were valued at "1" and therefore not significant. Duplicates were removed and duplicates experimental genes with no match in control were removed and only one valued at significant ("0") was kept and duplicate experimental genes with a match in control were removed and only one valued at non-significant ("1") was kept.
Read Files in topGO	Read in Experimental Data, Convert to a Data Frame and Assign First Colum Gene Name and Second Column P-Value	topGO Version 2.24.0	oneframe <- read.csv("X.csv", sep = ",") Vector1<- X1$Pvalue names(Vector1) <- X1$GeneName X =Experimental Sample
	Read in mappings	topGO Version 2.24.0	geneID2GO <- readMappings(file = "/Users/user/Documents/Anjeets_Data/Blast2go/X.txt", sep = "\t", IDsep = ",") X=read mapping for reference Species and Strain

Table 2: GO Enrichment Analysis (Continued)

Description	Task	Programme	Command
GO Enrichment Script	Run topGO	topGO Version 2.24.0	Sample1_GOdataMF <- new("topGOdata", ontology = "X", allGenes = Vector1, geneSel = topDiffGenes, nodeSize = 5, annot = annFUN.gene2GO, gene2GO = geneID2GO) X = the three different ontologies: Molecular Function (MF), Biological Process (BP) and Cellular Component (CC).
Run statistics on Enrichment Results	Run Fisher's exact statistical test with 'Classic', 'Elim', 'Weight' and 'Weight01' algorithms	topGO Version 2.24.0	resultFisherClassic <- runTest(GOdata, algorithm = "X", statistic = "fisher") X= "classic", "elim", "weight" or "weight01

Appendix 2: Variant statistics for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using high preservative concentrations and passaging the fastest growth (H/F) and evolution experiments using low preservative concentrations and passaging the slowest growth (L/S).

		Phenoxyethanol							
		Experiment				Control			
		1291	1292	1299	1318	1291	1292	1299	1318
H/F	UNFILTERED								
	SNPs	92	94		106	88	69	654	110
	Indels	13	14		9	11	15	135	10
	Total	105	108		115	99	84	789	120
	FILTERED								
	SNPs	4	5		8	5	1	61	8
	Indels	7	5		3	4	7	16	3
	Total	11	10		11	9	8	77	11
	snpEFF								
	SNPs	51	47		57	33	8	596	56
	Indels	67	45		32	36	72	183	36
	Total	118	92		89	69	80	779	92
	Without Controls								
	Total	50	0		19				

Appendix 2: Variant statistics for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using high preservative concentrations and passaging the fastest growth (H/F) and evolution experiments using low preservative concentrations and passaging the slowest growth (L/S) (Continued).

		Phenoxyethanol							
		Experiment				Control			
		1291	1292	1299	1318	1291	1292	1299	1318
L/S	UNFILTERED								
	SNPs	869	265	401	81604	246	161	141	96
	Indels	235	53	80	4701	28	23	42	9
	Total	1104	318	481	86305	274	184	183	105
	FILTERED								
	SNPs	86	19	32	8190	20	6	7	6
	Indels	20	8	15	432	5	8	11	5
	Total	106	27	47	8622	25	14	18	11
	snpEFF								
	SNPs	909	167	289	82638	179	57	81	48
	Indels	186	70	171	4637	38	71	548	55
	Total	1095	237	460	87275	217	128	629	103
	Without Controls								
	Total	244	38	118					

Appendix 2: Variant statistics for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using high preservative concentrations and passaging the fastest growth (H/F) and evolution experiments using low preservative concentrations and passaging the slowest growth (L/S) (Continued).

		Benzyl Alcohol							
		Experiment				Control			
		1291	1292	1299	1318	1291	1292	1299	1318
H/F	UNFILTERED								
	SNPs	73	232	86	216	62	101	942	95
	Indels	18	27	16	21	17	12	52	10
	Total	91	259	102	237	79	113	994	105
	FILTERED								
	SNPs	2	14	1	18	1	4	75	7
	Indels	7	12	11	6	6	6	22	3
	Total	9	26	12	24	7	10	97	10
	snpEFF								
	SNPs	20	141	10	194	15	38	753	36
	Indels	63	121	130	78	54	60	235	36
	Total	83	262	140	272	69	98	988	72
	Without Controls								
	Total	26	27	22	63				

Appendix 2: Variant statistics for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using high preservative concentrations and passaging the fastest growth (H/F) and evolution experiments using low preservative concentrations and passaging the slowest growth (L/S) (Continued).

		Benzyl Alcohol							
		Experiment				Control			
		1291	1292	1299	1318	1291	1292	1299	1318
	UNFILTERED								
	SNPs	287	179	5222		246	161	141	96
	Indels	20	39	191		28	23	42	9
	Total	307	218	5413		274	184	183	105
	FILTERED								
	SNPs	20	14	445		20	6	7	6
	Indels	10	8	67		5	8	11	5
	Total	30	22	512		25	14	18	11
L/S	snpEFF								
	SNPs	186	144	1825		179	57	81	48
	Indels	97	75	712		38	71	548	55
	Total	283	219	2537		217	128	629	103
	Without Controls								
	Total	86	91						

Appendix 2: Variant statistics for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using high preservative concentrations and passaging the fastest growth (H/F) and evolution experiments using low preservative concentrations and passaging the slowest growth (L/S) (Continued).

		BIT							
		Experiment				Control			
		1291	1292	1299	1318	1291	1292	1299	1318
	UNFILTERED								
	SNPs	71	62	110	128	62	101	942	95
	Indels	8	19	27	8	17	12	52	10
	Total	79	81	137	136	79	113	994	105
	FILTERED								
	SNPs	3	2	1	8	1	4	75	7
	Indels	5	5	12	6	6	6	22	3
	Total	8	7	13	14	7	10	97	10
H/F	snpEFF								
	SNPs	29	24	10	71	15	38	753	36
	Indels	36	45	137	69	54	60	235	36
	Total	65	69	147	140	69	98	988	72
	Without Controls								
	Total	17	0	25	12				

Appendix 2: Variant statistics for Bcc strains 1291, 1292, 1299 and 1318 before (control) and after preservative evolution experiments using high preservative concentrations and passaging the fastest growth (H/F) and evolution experiments using low preservative concentrations and passaging the slowest growth (L/S) (Continued).

		BIT							
		Experiment				Control			
		1291	1292	1299	1318	1291	1292	1299	1318
L/S	UNFILTERED								
	SNPs	307	858	740	2528	246	161	70	96
	Indels	54	126	76	319	28	23	22	9
	Total	361	984	816	2847	274	184	92	105
	FILTERED								
	SNPs	31	80	63	278	20	6	7	6
	Indels	5	14	17	7	5	8	11	5
	Total	36	94	80	285	25	14	18	11
	snpEFF								
	SNPs	247	721	526	2673	179	57	11	48
	Indels	50	136	198	59	38	71	119	55
	Total	297	857	724	2732	217	128	130	103
	Without Controls								
	Total	64	351	166	46				

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions.

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BA 1292	*acsA*	Acetyl-coenzyme A synthetase	Sythesis of Acetyl-CoA	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P39062
L/S BIT 1291	*acsA*	Acetyl-coenzyme A synthetase	Sythesis of Acetyl-CoA	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	P39062
L/S PH 1291	*acsA*	Acetyl-coenzyme A synthetase	Sythesis of Acetyl-CoA	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP INDELS	P39062
H/F BA 1291	*araG*	Arabinose import ATP-binding protein	Arabinose Import	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	SNP	P0AAF3
H/F PH 1291	*araG*	Arabinose import ATP-binding protein	Arabinose Import	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	SNP	P0AAF3

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
H/F BA 1291	*artI*	Putative ABC transporter arginine-binding protein 2	Amino Acid Transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	SNP	P30859
H/F PH 1291	*artI*	Putative ABC transporter arginine-binding protein 2	Amino Acid Transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	SNP	P30859
L/S BA 1292	*azoB*	NAD(P)H azoreductase	Azo Compounds Degradation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	Q8KU07
L/S PH 1291	*azoB*	NAD(P)H azoreductase	Azo Compounds Degradation	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	Q8KU07
H/F BA 1318	*bag*	IgA FC receptor	Antibody sensor	Cell Wall	Signalling	Synonymous variant	LOW	SNP	P27951
H/F BIT 1291	*bag*	IgA FC receptor	Antibody sensor	Cell Wall	Signalling	Synonymous variant	LOW	SNP	P27951
H/F BIT 1299	*bag*	IgA FC receptor	Antibody sensor	Cell Wall	Signalling	Synonymous variant	LOW	SNP	P27951
L/S BIT 1292	*bag*	IgA FC receptor	Antibody sensor	Cell Wall	Signalling	Synonymous variant	LOW	SNP	P27951

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S PH 1292	*bag*	IgA FC receptor	Antibody sensor	Cell Wall	Signalling	Synonymous variant	LOW	SNP	P27951
L/S PH 1299	*bag*	IgA FC receptor	Antibody sensor	Cell Wall	Signalling	Synonymous variant	LOW	SNP	P27951
H/F BA 1292	*ce*	Cellobiose 2-epimerase	Cabohydrate and Lactose Metabolism	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	F8WRK9
L/S BIT 1299	*ce*	Cellobiose 2-epimerase	Cabohydrate and Lactose Metabolism	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	F8WRK9
H/F BA 1292	*copA*	Copper-exporting P-type ATPase A	Copper and Lead Transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	INDELS	Q2FV64
L/S BIT 1299	*copA*	Copper-exporting P-type ATPase A	Copper and Lead Transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	SNP	Q2FV64
H/F BA 1299	*cugP*	UTP--glucose-1-phosphate uridylyltransferase	Glucose Biosynthesis	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P74285

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S PH 1299	*cugP*	UTP--glucose-1-phosphate uridylyltransferase	Glucose Biosynthesis	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P74285
L/S BA 1292	*cysK*	Cysteine synthase	Amino Acid Synthesis	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P63871
L/S PH 1291	*cysK*	Cysteine synthase	Amino Acid Synthesis	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	P63871
H/F BA 1291	*dmoA*	Dimethyl-sulfide monooxygenase	Dimethyl-sulphide Degredation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	E9JFX9
H/F PH 1291	*dmoA*	Dimethyl-sulfide monooxygenase	Dimethyl-sulphide Degredation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	E9JFX9
L/S BIT 1292	*dmoA*	Dimethyl-sulfide monooxygenase	Dimethyl-sulphide Degredation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	E9JFX9
H/F BA 1318	*dsbD*	Thiol:disulfide interchange protein	Protien Synthesis	Cell Wall	Metabolism	Upstream gene variant	MODIFIER	SNP	Q9JYM0

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BA 1291	*eno*	Enolase	Glycolysis	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	P37869
L/S BIT 1292	*eno*	Enolase	Glycolysis	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	P37869
H/F BA 1318	*ethA*	FAD-containing monooxygenase	Aromatic Keytone breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q7TVI2
H/F BIT 1291	*ethA*	FAD-containing monooxygenase	Aromatic Keytone breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q7TVI2
H/F BIT 1299	*ethA*	FAD-containing monooxygenase	Aromatic Keytone breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q7TVI2
L/S BIT 1292	*ethA*	FAD-containing monooxygenase	Aromatic Keytone breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q7TVI2
L/S PH 1292	*ethA*	FAD-containing monooxygenase	Aromatic Keytone breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q7TVI2

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S PH 1299	*ethA*	FAD-containing monooxygenase	Aromatic Keytone breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q7TVI2
H/F BA 1318	*eutB*	Ethanolamine ammonia-lyase heavy chain	Amino Acid Breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P0AEJ6
H/F PH 1318	*eutB*	Ethanolamine ammonia-lyase heavy chain	Amino Acid Breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P0AEJ6
H/F BA 1318	*eutC*	Ethanolamine ammonia-lyase light chain	Amino Acid Breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P19636
H/F PH 1318	*eutC*	Ethanolamine ammonia-lyase light chain	Amino Acid Breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P19636

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
H/F BA 1299	*fadA*	3-ketoacyl-CoA thiolase	Lipid Metabolism	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	O32177
L/S BIT 1299	*fadA*	3-ketoacyl-CoA thiolase	Lipid Metabolism	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	O32177
L/S PH 1299	*fadA*	3-ketoacyl-CoA thiolase	Lipid Metabolism	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	O32177
H/F BA 1299	*fdhD*	Sulfurtransferase	Sulphur Carrier	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	O87816
L/S PH 1299	*fdhD*	Sulfurtransferase	Sulphur Carrier	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	O87816
L/S BA 1292	*flhA*	Flagellar biosynthesis protein	Falgellum Component	Cell Wall	Flagellum	Upstream gene variant	MODIFIER	SNP	P40729
L/S PH 1291	*flhA*	Flagellar biosynthesis protein	Falgellum Component	Cell Wall	Flagellum	Upstream gene variant	MODIFIER	SNP	P40729
L/S BA 1292	*flhB*	Flagellar biosynthesis protein	Falgellum Component	Cell Wall	Flagellum	Upstream gene variant	MODIFIER	SNP	P76299
L/S PH 1291	*flhB*	Flagellar biosynthesis protein	Falgellum Component	Cell Wall	Flagellum	Upstream gene variant	MODIFIER	SNP	P76299

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BA 1291	*ftsB*	Cell division protein	Cell Cycle Protein	Cytoplasm	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q9KUJ3
L/S BIT 1292	*ftsB*	Cell division protein	Cell Cycle Protein	Cytoplasm	Normal Cell Function	Upstream gene variant	MODIFIER	SNP	Q9KUJ3
H/F BA 1299	*ggt*	Gamma-glutamyltranspeptidase	Amino Acid Synthesis	Secreted	Metabolism	Downstream gene variant	MODIFIER	SNP	P54422
H/F BIT 1291	*ggt*	Gamma-glutamyltranspeptidase	Amino Acid Synthesis	Secreted	Metabolism	Upstream gene variant	MODIFIER	SNP	P54422
L/S PH 1292	*ggt*	Gamma-glutamyltranspeptidase	Amino Acid Synthesis	Secreted	Metabolism	Downstream gene variant	MODIFIER	SNP	P54422
L/S PH 1299	*ggt*	Gamma-glutamyltranspeptidase	Amino Acid Synthesis	Secreted	Metabolism	Downstream gene variant	MODIFIER	SNP	P54422
H/F BIT 1299	*gshA*	Glutamate--cysteine ligase	Amino Acid Synthesis	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	P0A6W9
L/S BIT 1299	*gshA*	Glutamate--cysteine ligase	Amino Acid Synthesis	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	P0A6W9

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BA 1292	*hin*	DNA-invertase	Transcriptional activator/repressor	Cytoplasm	Flagellum	Upstream gene variant	MODIFIER	SNP	P03013
L/S BIT 1292	*hin*	DNA-invertase	Transcriptional activator/repressor	Cytoplasm	Flagellum	Upstream gene variant	MODIFIER	SNP	P03013
L/S PH 1291	*hin*	DNA-invertase	Transcriptional activator/repressor	Cytoplasm	Flagellum	Upstream gene variant/synonymous variant/missense variant	MODIFIER	SNP	P03013
L/S BIT 1299	*kpsM*	Polysialic acid transport protein	Polysialic acid transport	Cell Wall	Metabolism	Frameshift variant	HIGH	INDELS	P23889
L/S PH 1299	*kpsM*	Polysialic acid transport protein	Polysialic acid transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	INDELS	P23889

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BIT 1299	*kpsT*	Polysialic acid transport ATP-binding protein	Polysialic acid transport	Cell Wall	Metabolism	Upstream gene variant	MODIFIER	INDELS	P23888
L/S PH 1299	*kpsT*	Polysialic acid transport ATP-binding protein	Polysialic acid transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	INDELS	P23888
L/S BIT 1291	*melR*	Melibiose operon regulatory protein	Melibiose Metabolism Regulation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	P0ACH8
L/S BIT 1292	*melR*	Melibiose operon regulatory protein	Melibiose Metabolism Regulation	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	P0ACH8
L/S BIT 1299	*melR*	Melibiose operon regulatory protein	Melibiose Metabolism Regulation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	P0ACH8
L/S PH 1291	*melR*	Melibiose operon regulatory protein	Melibiose Metabolism Regulation	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	P0ACH8

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BIT 1299	*mfpsA*	Mannosylfructose-phosphate synthase	Cabohydrate Metabolism	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	A7TZT2
L/S PH 1299	*mfpsA*	Mannosylfructose-phosphate synthase	Cabohydrate Metabolism	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	A7TZT2
L/S PH 1299	*nadE*	Glutamine-dependent NAD(+) synthetase	NAD(+) Biosynthesis	Cytoplasm	Metabolism	Synonymous variant	LOW	SNP	Q03638
L/S BIT 1299	*nadE*	Glutamine-dependent NAD(+) synthetase	NAD(+) Biosynthesis	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q03638
H/F BA 1299	*ndh*	NADH dehydrogenase	Anerobic Respiration	Cytoplasm	Normal Cell Function	Upstream gene variant	MODIFIER	SNP	P00393
L/S PH 1299	*ndh*	NADH dehydrogenase	Anerobic Respiration	Cytoplasm	Normal Cell Function	Upstream gene variant	MODIFIER	SNP	P00393

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
H/F BA 1292	*nepI*	Purine ribonucleoside efflux pump	Purine Ribonucleoside Efflux	Cell Wall	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q8XGS2
H/F PH 1291	*nepI*	Purine ribonucleoside efflux pump	Purine Ribonucleoside Efflux	Cell Wall	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q8XGS3
L/S BA 1291	*nepI*	Purine ribonucleoside efflux pump	Purine Ribonucleoside Efflux	Cell Wall	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q8XGS2
L/S BA 1292	*nepI*	Purine ribonucleoside efflux pump	Purine Ribonucleoside Efflux	Cell Wall	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q8XGS4
L/S BIT 1291	*nepI*	Purine ribonucleoside efflux pump	Purine Ribonucleoside Efflux	Cell Wall	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q8XGS5
L/S BIT 1292	*nepI*	Purine ribonucleoside efflux pump	Purine Ribonucleoside Efflux	Cell Wall	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q8XGS2
L/S PH 1291	*nepI*	Purine ribonucleoside efflux pump	Purine Ribonucleoside Efflux	Cell Wall	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	Q8XGS2

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
H/F BA 1291	*occM*	Octopine transport system permease protein	Amino Acid Transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	SNP	P35114
H/F PH 1291	*occM*	Octopine transport system permease protein	Amino Acid Transport	Cell Wall	Metabolism	Downstream gene variant	MODIFIER	SNP	P35114
H/F BA 1299	*paaF*	2 2C3-dehydroadipyl-CoA hydratase	Phenylacetate Degradation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P76082
L/S PH 1299	*paaF*	2 2C3-dehydroadipyl-CoA hydratase	Phenylacetate Degradation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P76082
L/S BIT 1291	*pleD*	Response regulator	Cell Cycle Regulator	Cytoplasm	Stress Resistance	Downstream gene variant	MODIFIER	SNP	B8GZM2
L/S BIT 1299	*pleD*	Response regulator	Cell Cycle Regulator	Cytoplasm	Stress Resistance	Upstream gene variant	MODIFIER	SNP	B8GZM2

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BA 1292	*preA*	NAD-dependent dihydropyrimidine dehydrogenase subunit	Amino Acid Breakdown	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	P25889
L/S BIT 1292	*preA*	NAD-dependent dihydropyrimidine dehydrogenase subunit	Amino Acid Breakdown	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P25889
L/S BIT 1292	*pyrG*	CTP synthase	Amino Acid Regulation	Cytoplasm	Metabolism	Missense variant	MODERATE	SNP	P0A7E5
L/S BA 1291	*pyrG*	CTP synthase	Amino Acid Regulation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P0A7E5
H/F BA 1291	*rbsB*	Ribose import binding protein	Ribose Import	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	P44737
H/F PH 1291	*rbsB*	Ribose import binding protein	Ribose Import	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	SNP	P44737

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S BA 1291	*ribBA*	Riboflavin biosynthesis protein	Riboflavin Synthesis	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	A5U2B7
L/S BIT 1299	*ribBA*	Riboflavin biosynthesis protein	Riboflavin Synthesis	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	A5U2B7
L/S BA 1291	*rlmB*	23S rRNA (guanosine-2'-O-)-methyltransferase	Ribose Methylation	Cytoplasm	Normal Cell Function	Upstream gene variant	MODIFIER	SNP INDELS	P63177
L/S BIT 1292	*rlmB*	23S rRNA (guanosine-2'-O-)-methyltransferase	Ribose Methylation	Cytoplasm	Normal Cell Function	Upstream gene variant	MODIFIER	INDELS	P63177
H/F BA 1299	*rutR*	HTH-type transcriptional regulator	Amino Acid Regulation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	P0ACU2
L/S BA 1292	*rutR*	HTH-type transcriptional regulator	Amino Acid Regulation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	INDELS	P0ACU2

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Experimental Condition	Gene	Protein	Description	Location	Classification	Effect	Effect Impact	Variant Type	Uniprot KB Number
L/S PH 1299	*rutR*	HTH-type transcriptional regulator	Amino Acid Regulation	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	P0ACU2
L/S PH 1299	*tld*	GDP-6-deoxy-D-talose 4-dehydrogenase	LPS Biosynthesis	Cell Wall	Cell Membrane	Frameshift variant	HIGH	INDELS	Q9JRN7
L/S BIT 1299	*tld*	GDP-6-deoxy-D-talose 4-dehydrogenase	LPS Biosynthesis	Cell Wall	Cell Membrane	Downstream gene variant	MODIFIER	INDELS	Q9JRN7
L/S BA 1292	*ttuB*	Putative tartrate transporter	Tartrate Transporter	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	Q44470
L/S BIT 1299	*ttuB*	Putative tartrate transporter	Tartrate Transporter	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	Q44470
H/F BA 1292	*yycN*	putative N-acetyltransferase	Unknown Transferase	Cytoplasm	Metabolism	Upstream gene variant	MODIFIER	INDELS	O32293
L/S BIT 1299	*yycN*	putative N-acetyltransferase	Unknown Transferase	Cytoplasm	Metabolism	Downstream gene variant	MODIFIER	SNP	O32293

Appendix 3: Table of SNP/INDEL information for genes in two or more experimental conditions involved in normal cellular functions (Continued).

Abbreviations:
L/S, Low and Slow Methodology
H/F, High and Fast Methodology
BIT, Benzisothiazolinone
PH, Phenoxyethanol
BA, Benzyl Alcohol
1291, *B. cenocepacia* strain 1291
1292, *B. cenocepacia* strain 1292
1299, *B. lata* strain 1299
1318, *B. cenocepacia* strain 1318

Appendix 4: GO Enrichment Statistics Generated From topGO

The total number of annotated topGO results for all experiments

Table 1: Total number of all annotated GO terms for experimental conditions

Bcc Strain		Preservative and Method					
		Phenoxyethanol		BIT		Benzyl Alcohol	
		H/F	L/S	H/F	L/S	H/F	L/S
	1291	21	140	7	48	17	107
	1292	N/A	40	N/A	197	19	96
	1299		68	18	74	20	
	1318	18		21	33	43	

The total number of significant topGO results for all experiments using Fishers statistical method and various algorithm

For the classic method a total 117 significant GO terms were seen over 12 experimental conditions. Removing duplicate terms, there were 76 unique GO Terms.

For the Elim method a total of 85 significant GO terms were seen over 12 experimental conditions. Removing duplicate terms, there were 76 unique GO terms.

For the Weight method a total of 40 significant GO terms were seen over 12 experimental conditions. Removing duplicate terms, there were 30 unique GO terms.

For the Weight01 method a total of 36 significant GO terms were seen over 11 experimental conditions. Removing duplicate terms, there were 27 unique GO terms.

Table 2: Total number of all significant GO terms for experimental conditions using the Fishers statistical method and Classic, Elim, Weight and Weight01 algorithm.

Algorithm and Bcc Strain		Preservative and Method					
		Phenoxyethanol		BIT		Benzyl Alcohol	
		H/F	L/S	H/F	L/S	H/F	L/S
Classic	1291	1	24	2	19	1	20
	1292	N/A	0	N/A	24	3	0
	1299		11	0	10	0	
	1318	1		0	1	0	
Elim	1291	1	15	2	7	1	18
	1292	N/A	0	N/A	19	3	0
	1299		7	0	10	0	
	1318	1		0	1	0	
Weight	1291	1	5	2	4	1	6
	1292	N/A	0	N/A	6	2	0
	1299		5	0	5	1	
	1318	2		0	0	0	
Weight01	1291	1	2	2	3	1	1
	1292	N/A	0	N/A	4	1	0
	1299		5	0	12	0	
	1318	4		0	0	0	

Abbreviations:
L/S, Low and Slow Methodology
H/F, High and Fast Methodology
BIT, Benzisothiazolinone
PH, Phenoxyethanol
BA, Benzyl Alcohol
1291, *B. cenocepacia* strain 1291
1292, *B. cenocepacia* strain 1292
1299, *B. lata* strain 1299
1318, *B. cenocepacia* strain 1318

Appendix 5: GO terms and experimental conditions shared with all statistical algorithms

Statistical Algorithm	Experiment	GO Type	GO Number	GO Description	Annotated	Significant	Expected	P Value
Classic	L/S PH 1299	BP	GO:0019439	Aromatic compound catabolic process	8	5	0.83	0.00049
Elim	L/S PH 1299	BP	GO:0019439	Aromatic compound catabolic process	8	5	0.83	0.00045
Weight	L/S PH 1299	BP	GO:0019439	Aromatic compound catabolic process	8	5	0.83	0.00049
Weight01	L/S PH 1299	BP	GO:0019439	Aromatic compound catabolic process	8	5	0.83	0.00045
Classic	L/S PH 1291	MF	GO:0005524	Atp binding	12	12	8.98	0.0275
Classic	L/S BA 1291	MF	GO:0005524	Atp binding	5	5	2.65	0.038
Elim	L/S PH 1291	MF	GO:0005524	Atp binding	12	12	8.98	0.0211
Elim	L/S BA 1291	MF	GO:0005524	Atp binding	5	5	2.65	0.038
Weight	L/S PH 1291	MF	GO:0005524	Atp binding	12	12	8.98	0.0275
Weight	L/S BA 1291	MF	GO:0005524	Atp binding	5	5	2.65	0.038
Weight01	L/S PH 1291	MF	GO:0005524	Atp binding	12	12	8.98	0.021
Weight01	L/S BA 1291	MF	GO:0005524	Atp binding	5	5	2.65	0.038
Classic	L/S PH 1299	BP	GO:0042537	Benzene-containing compound metabolic process	5	5	0.52	1.10E-05
Elim	L/S PH 1299	BP	GO:0042537	Benzene-containing compound metabolic process	5	5	0.52	1.00E-05
Weight	L/S PH 1299	BP	GO:0042537	Benzene-containing compound metabolic process	5	5	0.52	1.10E-05
Weight01	L/S PH 1299	BP	GO:0042537	Benzene-containing compound metabolic process	5	5	0.52	1.00E-05
Classic	L/S PH 1299	BP	GO:0070887	Cellular response to chemical stimulus	11	6	1.15	0.00033
Elim	L/S PH 1299	BP	GO:0070887	Cellular response to chemical stimulus	11	6	1.15	0.0003
Weight	L/S PH 1299	BP	GO:0070887	Cellular response to chemical stimulus	11	6	1.15	0.0001
Weight01	L/S PH 1299	BP	GO:0070887	Cellular response to chemical stimulus	11	6	1.15	9.60E-05

Appendix 5: GO terms and experimental conditions shared with all statistical algorithms (Continued)

Statistical Algorithm	Experiment	GO Type	GO Number	GO Description	Annotated	Significant	Expected	P Value
Classic	L/S PH 1291	CC	GO:0005737	Cytoplasm	12	11	7.69	0.027
Classic	L/S BIT 1292	CC	GO:0005737	Cytoplasm	44	41	33.14	0.00059
Classic	L/S BA 1291	CC	GO:0005737	Cytoplasm	9	8	4.42	0.011
Elim	L/S PH 1291	CC	GO:0005737	Cytoplasm	12	11	7.69	0.017
Elim	L/S BIT 1292	CC	GO:0005737	Cytoplasm	44	41	33.14	0.00059
Elim	L/S BA 1291	CC	GO:0005737	Cytoplasm	9	8	4.42	0.011
Weight	L/S PH 1291	CC	GO:0005737	Cytoplasm	12	11	7.69	0.027
Weight	L/S BIT 1292	CC	GO:0005737	Cytoplasm	44	41	33.14	0.00059
Weight	L/S BA 1291	CC	GO:0005737	Cytoplasm	9	8	4.42	0.011
Weight01	L/S PH 1291	CC	GO:0005737	Cytoplasm	12	11	7.69	0.017
Weight01	L/S BIT 1292	CC	GO:0005737	Cytoplasm	44	41	33.14	0.016
Weight01	L/S BA 1291	CC	GO:0005737	Cytoplasm	9	8	4.42	0.011
Classic	L/S BA 1291	BP	GO:0009064	Glutamine family amino acid metabolic process	5	5	2.47	0.0267
Elim	L/S BA 1291	BP	GO:0009064	Glutamine family amino acid metabolic process	5	5	2.47	0.0267
Weight	L/S BA 1291	BP	GO:0009064	Glutamine family amino acid metabolic process	5	5	2.47	0.027
Weight01	L/S BA 1291	BP	GO:0009064	Glutamine family amino acid metabolic process	5	5	2.47	0.027
Classic	L/S PH 1291	MF	GO:1901363	Heterocyclic compound binding	99	83	74.12	0.0025
Classic	L/S BIT 1291	MF	GO:1901363	Heterocyclic compound binding	40	24	16.87	0.0015
Elim	L/S PH 1291	MF	GO:1901363	Heterocyclic compound binding	99	83	74.12	0.018
Elim	L/S BIT 1291	MF	GO:1901363	Heterocyclic compound binding	40	24	16.87	0.0015
Elim	L/S BIT 1292	MF	GO:1901363	Heterocyclic compound binding	122	101	95.06	0.04
Weight	L/S PH 1291	MF	GO:1901363	Heterocyclic compound binding	99	83	74.12	0.0086

Appendix 5: GO terms and experimental conditions shared with all statistical algorithms (Continued)

Statistical Algorithm	Experiment	GO Type	GO Number	GO Description	Annotated	Significant	Expected	P Value
Weight	L/S BIT 1291	MF	GO:1901363	Heterocyclic compound binding	40	24	16.87	0.0015
Weight01	H/F PH 1318	MF	GO:1901363	Heterocyclic compound binding	31	8	6.1	0.03
Weight01	L/S BIT 1291	MF	GO:1901363	Heterocyclic compound binding	40	24	16.87	0.011
Weight01	L/S BIT 1299	MF	GO:1901363	Heterocyclic compound binding	268	42	48.81	0.0018
Classic	H/F PH 1291	MF	GO:0016787	Hydrolase activity	17	9	5.51	0.04
Classic	L/S BA 1291	MF	GO:0016787	Hydrolase activity	28	20	14.82	0.018
Elim	H/F PH 1291	MF	GO:0016787	Hydrolase activity	17	9	5.51	0.04
Elim	L/S BA 1291	MF	GO:0016787	Hydrolase activity	28	20	14.82	0.018
Weight	H/F PH 1291	MF	GO:0016787	Hydrolase activity	17	9	5.51	0.04
Weight	H/F BA 1292	MF	GO:0016787	Hydrolase activity	16	6	3.35	0.031
Weight	L/S BA 1291	MF	GO:0016787	Hydrolase activity	28	20	14.82	0.018
Weight01	H/F PH 1291	MF	GO:0016787	Hydrolase activity	17	9	5.51	0.04
Classic	L/S BIT 1299	CC	GO:0016021	Integral component of membrane	207	37	30.99	0.039
Elim	L/S BIT 1299	CC	GO:0016021	Integral component of membrane	207	37	30.99	0.039
Elim	H/F BIT 1292	CC	GO:0016021	Integral component of membrane	29	14	9.98	0.0285
Weight	L/S BIT 1299	CC	GO:0016021	Integral component of membrane	207	37	30.99	0.039
Weight01	L/S BIT 1299	CC	GO:0016021	Integral component of membrane	207	37	30.99	0.039

Appendix 5: GO terms and experimental conditions shared with all statistical algorithms (Continued)

Statistical Algorithm	Experiment	GO Type	GO Number	GO Description	Annotated	Significant	Expected	P Value
Classic	L/S BIT 1299	BP	GO:0032259	Methylation	26	9	4.57	0.0271
Elim	L/S BIT 1299	BP	GO:0032259	Methylation	26	9	4.57	0.0255
Weight	L/S BIT 1299	BP	GO:0032259	Methylation	26	9	4.57	0.0271
Weight01	L/S BIT 1299	BP	GO:0032259	Methylation	26	9	4.57	0.0255
Classic	L/S BIT 1299	MF	GO:0008168	Methyltransferase activity	26	9	4.73	0.03354
Elim	L/S BIT 1299	MF	GO:0008168	Methyltransferase activity	26	9	4.73	0.0318
Weight	L/S BIT 1299	MF	GO:0008168	Methyltransferase activity	26	9	4.73	0.0335
Weight01	L/S BIT 1299	MF	GO:0008168	Methyltransferase activity	26	9	4.73	0.0056
Classic	L/S PH 1299	BP	GO:0072329	Monocarboxylic acid catabolic process	5	5	0.52	1.10E-05
Elim	L/S PH 1299	BP	GO:0072329	Monocarboxylic acid catabolic process	5	5	0.52	1.00E-05
Weight	L/S PH 1299	BP	GO:0072329	Monocarboxylic acid catabolic process	5	5	0.52	1.10E-05
Weight01	L/S PH 1299	BP	GO:0072329	Monocarboxylic acid catabolic process	5	5	0.52	1.00E-05
Classic	L/S PH 1291	MF	GO:0097159	Organic cyclic compound binding	99	83	74.12	0.0025
Classic	L/S BIT 1291	MF	GO:0097159	Organic cyclic compound binding	40	24	16.87	0.0015
Elim	L/S PH 1291	MF	GO:0097159	Organic cyclic compound binding	99	83	74.12	0.018
Elim	L/S BIT 1291	MF	GO:0097159	Organic cyclic compound binding	40	24	16.87	0.0015
Elim	L/S BIT 1292	MF	GO:0097159	Organic cyclic compound binding	122	101	95.06	0.04
Weight	L/S PH 1291	MF	GO:0097159	Organic cyclic compound binding	99	83	74.12	0.0086
Weight	L/S BIT 1291	MF	GO:0097159	Organic cyclic compound binding	40	24	16.87	0.0015
Weight01	H/F PH 1318	MF	GO:0097159	Organic cyclic compound binding	31	8	6.1	0.03
Weight01	L/S BIT 1291	MF	GO:0097159	Organic cyclic compound binding	40	24	16.87	0.011
Weight01	L/S BIT 1299	MF	GO:0097159	Organic cyclic compound binding	268	42	48.81	0.0018

Appendix 5: GO terms and experimental conditions shared with all statistical algorithms (Continued)

Statistical Algorithm	Experiment	GO Type	GO Number	GO Description	Annotated	Significant	Expected	P Value
Classic	L/S PH 1299	BP	GO:1901361	Organic cyclic compound catabolic process	8	5	0.83	0.00049
Elim	L/S PH 1299	BP	GO:1901361	Organic cyclic compound catabolic process	8	5	0.83	0.00045
Weight	L/S PH 1299	BP	GO:1901361	Organic cyclic compound catabolic process	8	5	0.83	0.00049
Weight01	L/S PH 1299	BP	GO:1901361	Organic cyclic compound catabolic process	8	5	0.83	0.00045
Classic	L/S BIT 1299	BP	GO:0055114	Oxidation-reduction process	114	27	20.06	0.0465
Classic	H/F BIT 1291	BP	GO:0055114	Oxidation-reduction process	14	6	2.52	0.013
Elim	L/S BIT 1299	BP	GO:0055114	Oxidation-reduction process	114	27	20.06	0.0411
Elim	H/F BIT 1291	BP	GO:0055114	Oxidation-reduction process	14	6	2.52	0.0066
Weight	L/S BIT 1299	BP	GO:0055114	Oxidation-reduction process	114	27	20.06	0.0465
Weight	H/F BIT 1291	BP	GO:0055114	Oxidation-reduction process	14	6	2.52	0.013
Weight01	H/F BIT 1291	BP	GO:0055114	Oxidation-reduction process	14	6	2.52	0.0066
Classic	L/S BIT 1299	MF	GO:0016491	Oxidoreductase activity	117	29	21.31	0.03444
Classic	H/F BIT 1291	MF	GO:0016491	Oxidoreductase activity	14	6	3.16	0.049
Elim	H/F BIT 1291	MF	GO:0016491	Oxidoreductase activity	14	6	3.16	0.032
Weight	H/F BIT 1291	MF	GO:0016491	Oxidoreductase activity	14	6	3.16	0.049
Weight01	H/F BIT 1291	MF	GO:0016491	Oxidoreductase activity	14	6	3.16	0.032
Classic	H/F PH 1318	MF	GO:0036094	Small molecule binding	14	6	2.75	0.022
Classic	L/S PH 1291	MF	GO:0036094	Small molecule binding	18	17	13.48	0.0321
Classic	L/S BIT 1291	MF	GO:0036094	Small molecule binding	6	5	2.53	0.0456
Classic	L/S BA 1291	MF	GO:0036094	Small molecule binding	9	8	4.76	0.024
Elim	H/F PH 1318	MF	GO:0036094	Small molecule binding	14	6	2.75	0.022

Appendix 5: GO terms and experimental conditions shared with all statistical algorithms (Continued)

Statistical Algorithm	Experiment	GO Type	GO Number	GO Description	Annotated	Significant	Expected	P Value
Elim	L/S PH 1291	MF	GO:0036094	Small molecule binding	18	17	13.48	0.023
Elim	L/S BIT 1291	MF	GO:0036094	Small molecule binding	6	5	2.53	0.0456
Elim	L/S BA 1291	MF	GO:0036094	Small molecule binding	9	8	4.76	0.024
Weight	H/F PH 1318	MF	GO:0036094	Small molecule binding	14	6	2.75	0.017
Weight	L/S BIT 1291	MF	GO:0036094	Small molecule binding	6	5	2.53	0.0456
Weight01	H/F PH 1318	MF	GO:0036094	Small molecule binding	14	6	2.75	0.024
Weight01	L/S BIT 1291	MF	GO:0036094	Small molecule binding	6	5	2.53	0.046
Classic	L/S BIT 1292	MF	GO:0003735	Structural constituent of ribosome	12	12	9.35	0.047
Elim	L/S BIT 1292	MF	GO:0003735	Structural constituent of ribosome	12	12	9.35	0.042
Weight	L/S BIT 1292	MF	GO:0003735	Structural constituent of ribosome	12	12	9.35	0.047
Weight01	L/S BIT 1292	MF	GO:0003735	Structural constituent of ribosome	12	12	9.35	0.042
Classic	L/S BIT 1299	MF	GO:0046914	Transition metal ion binding	20	8	3.64	0.01818
Elim	L/S BIT 1299	MF	GO:0046914	Transition metal ion binding	20	8	3.64	0.0173
Weight	L/S BIT 1299	MF	GO:0046914	Transition metal ion binding	20	8	3.64	0.0182
Weight01	L/S BIT 1299	MF	GO:0046914	Transition metal ion binding	20	8	3.64	0.0194
Classic	H/F BA 1292	BP	GO:0006810	Transport	21	7	3.8	0.042
Elim	H/F BA 1292	BP	GO:0006810	Transport	21	7	3.8	0.042
Elim	H/F BIT 1292	BP	GO:0006810	Transport	14	10	4.85	0.003
Weight	H/F BA 1292	BP	GO:0006810	Transport	21	7	3.8	0.042
Weight01	H/F BA 1292	BP	GO:0006810	Transport	14	10	4.85	0.032

Appendix 5: GO terms and experimental conditions shared with all statistical algorithms (Continued)

Statistical Algorithm	Experiment	GO Type	GO Number	GO Description	Annotated	Significant	Expected	P Value
Classic	H/F BA 1291	MF	GO:0005215	Transporter activity	11	6	2.34	0.0079
Elim	H/F BA 1291	MF	GO:0005215	Transporter activity	11	6	2.34	0.0079
Weight	H/F BA 1291	MF	GO:0005215	Transporter activity	11	6	2.34	0.0079
Weight	H/F BA 1299	MF	GO:0005215	Transporter activity	79	5	2.39	0.05
Weight01	H/F BA 1291	MF	GO:0005215	Transporter activity	11	6	2.34	0.0079

Abbreviations:
L/S, Low and Slow Methodology
H/F, High and Fast Methodology
BIT, Benzisothiazolinone
PH, Phenoxyethanol
BA, Benzyl Alcohol
1291, *B. cenocepacia* strain 1291
1292, *B. cenocepacia* strain 1292
1299, *B. lata* strain 1299
1318, *B. cenocepacia* strain 1318