Understanding Bacterial Resistance and Dissemination: The Impact of Biocide Priming

A thesis submitted in part fulfilment for the degree of Doctor of Philosophy

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Abstract

**Background:** Public awareness to infection control, for example the current covid-19 pandemic, can cause a rise in the use of biocidal products. Uncontrolled use of biocides, especially at sub-inhibitory concentrations has led to growing concerns that their selective pressure may favour the prevalence of less susceptible bacterial strains and encourage the expression and dissemination of antibiotic resistance genes.

**Aims:** To investigate the priming effects of sub-inhibitory concentrations of biocides on antibiotic resistance in bacteria and to understand the resistance mechanisms activated by these effects.

**Methodology:** *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* were exposed to sub-inhibitory concentrations of biocides (hydrogen peroxide, chlorhexidine, glutaraldehyde and benzalkonium chloride). Minimum inhibitory concentrations of antibiotics and biocides were determined before and after exposure to low concentrations of biocides to test for changes in biocide tolerance and cross resistance to antibiotics. Efflux pump inhibitors (thioridazine and chlorpromazine) were used to investigate efflux as a mechanism. Known antibiotic resistance genes were sequenced to look for any mutations and Quantitative Realtime PCR was used to compare their regulation and expression between parent and primed strains. Growth assays were used to investigate if there were fitness costs attached to the antibiotic cross resistance observed.

**Results:** Stable cross-resistance to antibiotics was observed in bacterial strains, with no observed increased tolerance to biocides. Six-fold increases in MIC to cephalothin and four-fold to ceftriaxone and ampicillin were observed in hydrogen peroxide primed *E. coli* and thioridazine increased the susceptibility of *E. coli* to cephalothin and cefoxitin. The *ompF* porin gene was downregulated by 30.5-fold after exposure to hydrogen peroxide and a further 37.9-fold when grown in 4 mg/l cephalothin compared to parent strain. Chlorhexidine primed *S. aureus* showed a four-fold increase in MIC to oxacillin, both thioridazine and chlorpromazine increased susceptibility to oxacillin. Sequence analysis of *norA* and the *norA* promoter region showed a single adenine to thymine change in codon 313, corresponding to isoleucine to phenylalanine change in position 313 in the coding region, but no change in the promoter region. Gene expression revealed a seven-fold upregulation in *norA* efflux gene. Glutaraldehyde primed *P. aeruginosa* showed a four-fold increased MIC to sulphatriad and an eight-fold increased MIC to ciprofloxacin. No increased expression of the efflux gene *mexJ* and its regulator *mexL* was observed, suggesting efflux may not be the responsible mechanism of cross-resistance observed in *P. aeruginosa*. There was no observable fitness cost as a result of the cross-resistance to antibiotics in the strains studied.

**Conclusion:** The widespread and uncontrolled use of biocides may exacerbate the ongoing antibiotic resistance that is seen in clinically relevant bacteria such as *E. coli, S. aureus* and *P. aeruginosa*. Sub-inhibitory use of biocides may trigger the activation of protective response in bacteria that can lead to antibiotic cross-resistance. The results shown in EcH2O2 and PaGTA support the theory that not all antibiotic resistant phenotypes come at a cost to fitness because bacteria sometimes have trade-offs and other beneficial mutations that compensates for or offset imbalances that may occur from a selective resistance. The reduced survival of SaCHG in water shows the oxacillin résistance came at a cost.
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To my wonderful husband Tim Adkin and beautiful daughters Emily and Laura, thank you so much for your patience and support all through these four years, as without it this PhD would not have been possible.
Declaration

The composition and experiments contained in this thesis are the work of the author unless otherwise stated. No portion of the work referred to in this thesis has been submitted in support of an application of another degree.
Publications and Presentations

Publications:


Presentations

28th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID)
Madrid Spain (21-24 April 2018):

Poster presented: Antibiotic cross-resistance in Escherichia coli and Staphylococcus aureus after exposure to biocides.
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BKC</td>
<td>Benzalkonium chloride</td>
</tr>
<tr>
<td>CHG</td>
<td>Chlorhexidine</td>
</tr>
<tr>
<td>CPZ</td>
<td>Chlorpromazine</td>
</tr>
<tr>
<td>EcH2O2</td>
<td>(H_2O_2) primed <em>Escherichia coli</em></td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on antimicrobial susceptibility testing</td>
</tr>
<tr>
<td>GTA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>PaATCC</td>
<td><em>Pseudomonas aeruginosa</em> ATCC</td>
</tr>
<tr>
<td>PaGTA</td>
<td>Glutaraldehyde primed <em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PaBKC</td>
<td>Benzalkonium chloride primed <em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>SaATCC</td>
<td><em>Staphylococcus aureus</em> ATCC</td>
</tr>
<tr>
<td>SaCHG</td>
<td>Chlorhexidine primed <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>TZ</td>
<td>Thioridazine</td>
</tr>
</tbody>
</table>
1. Chapter One: General introduction.................................................................23

1.1 Introduction ..................................................................................................23

1.2 Selected biocides and their mode of action .................................................26

1.2.1 Hydrogen peroxide ..................................................................................28

1.2.2 Chlorhexidine ..........................................................................................31

1.2.3 Benzalkonium chloride ...........................................................................31

1.2.4 Glutaraldehyde .......................................................................................32

1.3 Bacterial tolerance to biocides .....................................................................33

1.3.1 Biofilms ..................................................................................................34

1.3.2 Upregulation of efflux pumps .................................................................35

1.3.3 Degradation and inactivation of biocides ................................................37

1.3.4 Modification of target sites .....................................................................37

1.3.5 Impermeability .........................................................................................38

1.4 Evidence of biocide-antibiotic cross-resistance .........................................40

1.5 Gap in current knowledge ...........................................................................45
1.6. Aims and objectives of this study ......................................................... 47

2. Chapter Two: priming with biocides: a pathway to antibiotic resistance ............. 49

2.1. Introduction .............................................................................................. 49

2.1.1. Chapter objectives .................................................................................. 52

2.2. Methods ..................................................................................................... 54

2.2.1. Bacterial strains, storage and growth conditions .................................... 54

2.2.2. Bacterial growth media and reagents ..................................................... 54

2.2.3. Biocides and concentration ranges ....................................................... 55

2.2.4. Antibiotics and concentration ranges for minimum inhibitory concentration test ........................................................................................................ 56

2.2.5. Preparation of working solutions for minimum inhibitory concentration experiments. 57

2.2.6. Preparation of inoculum Minimum inhibitory concentration and antibiotic susceptibility experiments ............................................................... 62

Colony suspension method ........................................................................... 62

2.2.7. Establishing minimum inhibitory concentration of biocides ................... 62
2.2.8. Antibiotic susceptibility testing using disc diffusion test

2.2.9. Priming of bacteria strains with biocides

Stepwise method

Gradient plate method

2.2.10. Testing for increased tolerance to biocides

2.2.11. Stability of adaptive resistance and tolerance

2.2.12. Biochemical tests of stable isolates

Gram staining

Analytical profile index 20E (API20E) test

Growth of organisms in cetrimide agar

Oxidase test

Catalase test

Coagulase test

2.2.13. Genomic DNA extraction

2.2.14. Measurement of DNA concentration
2.2.15. Polymerase chain reaction (PCR) ........................................... 71

Cycling parameters ........................................................................... 72

2.2.16. Agarose gel electrophoresis ................................................... 73

2.2.17. 16S rRNA sequencing ............................................................ 73

2.3. Results ....................................................................................... 74

2.3.1. Establishing minimum inhibitory concentration of biocides .......... 74

2.3.2. Testing for increased tolerance to biocides ................................ 74

2.3.3. Testing for cross-resistance to antibiotics using disc diffusion test .... 76

2.3.4. Stability of adaptive resistance and tolerance ............................ 79

2.3.5. Minimum inhibitory concentration of antibiotics ...................... 79

2.3.6. Biochemical tests of stable isolates .......................................... 82

2.3.7. PCR and 16S rRNA sequencing .............................................. 83

2.4. Discussion ................................................................................. 85

2.4.1. The effect of biocide priming ................................................. 88

2.5. Conclusion ................................................................................. 90
3. Chapter Three: Analysis of efflux mechanism as a contributor to adaptive resistance to antibiotics ................................................................................................................................. 91

3.1. Introduction .................................................................................................................. 91

3.1.1. Enzymatic inactivation of drugs ............................................................................. 91

3.1.2. Effect of cell envelope .......................................................................................... 92

3.1.3. Active drug removal ............................................................................................... 93

3.1.4. Efflux pump expression ......................................................................................... 97

3.1.5. Mechanisms of efflux pump inhibitors ............................................................... 98

3.1.6. Disadvantages of efflux pump inhibitors ............................................................ 99

3.1.7. Chapter objectives ............................................................................................... 101

3.2 Methods ..................................................................................................................... 102

3.2.1. Antibiotics and chemicals .................................................................................... 102

3.2.2. Preparation of antibiotic solutions for minimum inhibitory concentration experiments .......................................................................................................................... 103

3.2.3. AmpC β-lactamase test ....................................................................................... 104

3.2.4. Preparation ethidium bromide agar plates for testing efflux activity .... 106
3.2.5. Establishing MIC of efflux pump inhibitors TZ and CPZ .......................... 108

3.2.6. Effect of efflux pump inhibitors ............................................................... 108

3.3. Results .............................................................................................................. 111

3.3.1. AmpC β-lactamase test ............................................................................... 111

3.3.2. Ethidium bromide agar (Cartwheel method) of assessing efflux. .......... 113

3.3.3. Establishing MIC of efflux pump inhibitors ............................................... 113

3.3.4. Effect of efflux pump inhibitors on bacterial resistance ......................... 115

3.3.5. The effect of TZ on the resistance of EcH2O2 to antibiotics .................... 115

3.3.6. The effect of TZ and CPZ on SaCHG resistance to oxacillin ................. 116

3.3.7. The effect of TZ on PaGTA resistance to ciprofloxacin ............................. 117

3.3.8. The effect of TZ on PaBKC tolerance to benzalkonium chloride ............ 119

3.4. Discussion ........................................................................................................ 121

3.4.1. β-lactamase test ......................................................................................... 121

3.4.2. Ethidium bromide-agar (Cartwheel) method. ......................................... 122

3.4.3. Effect of efflux pump inhibitors ................................................................. 123
cDNA synthesis for Realtime PCR ................................................................. 143

4.2.7. Quantitative Realtime PCR (qPCR) ......................................................... 145

Quantification of relative gene expression ....................................................... 147

4.3. Results ........................................................................................................ 148

4.3.1. Primer design .......................................................................................... 148

4.3.2. Polymerase chain reaction (PCR) and gene sequence analysis .......... 149

The mutational effects if sub-inhibitory concentrations of hydrogen peroxide on *E. coli* stress response and regulatory
genesis ............................................................................................................. 149

The mutational effects if sub-inhibitory concentrations of chlorhexidine on *S. aureus*
norA and lmrS efflux genes genes .................................................................... 152

The mutational effects if sub-inhibitory concentrations of glutaraldehyde on *P. aeruginosa* efflux gene mexJ and its repressor mexL ......................................................... 154

4.3.3. Quantitative Realtime PCR ................................................................... 156

Realtime PCR primers design ........................................................................... 156

Housekeeping gene selection and validation ..................................................... 156
Gene and primer efficiency ........................................................................................................................................157

The effect of sub-inhibitory concentrations of hydrogen peroxide on the expression of *E. coli* oxidative stress response and regulatory genes ..............................................................................................................................159

The effect of sub-inhibitory concentrations of chlorhexidine on the expression of *S. aureus* norA and *Imr* efflux genes ..............................................................................................................................................162

The effect of sub-inhibitory concentrations of glutaraldehyde on the expression of *P. aeruginosa* efflux gene mexJ and its regulator mexL .........................................................................................................................164

4.4. Discussion .........................................................................................................................................................166

The effect of sub-inhibitory concentrations of hydrogen peroxide on the expression of *E. coli* oxidative stress response and regulatory genes ..............................................................................................................................166

The effect of sub-inhibitory concentrations of hydrogen peroxide on *ompF* gene expression in EcH2O2.................................................................................................................................................................168

The effect of sub-inhibitory concentration of hydrogen peroxide on *acrA* gene expression in EcH2O2.................................................................................................................................................................170

The effect of sub-inhibitory concentrations of chlorhexidine on the expression of *S. aureus* norA and *lmr* efflux genes ..............................................................................................................................................172
The effect of sub-inhibitory concentrations of glutaraldehyde on the expression of *P. aeruginosa* efflux gene *mexJ* and its regulator *mexL* .................................................................175

5. Chapter Five: Fitness cost of antibiotic resistance ..................................................178

5.1. Introduction ...........................................................................................................178

5.1.1. Chapter objectives .........................................................................................181

5.2 Methods ...............................................................................................................183

5.2.1 Bacterial strains, storage and growth conditions ...........................................183

5.2.2 Reagents and chemicals ..................................................................................183

5.2.3 Preparation of inoculum ..................................................................................183

5.2.4 Comparison of growth of parent and tolerant strains .......................................183

5.2.5 Biofilm growth with crystal violet .....................................................................184

5.2.6 Biofilm formation in 2% glucose .....................................................................185

5.2.7 Survival in water ...............................................................................................185

5.2.8 Spiral plate method ..........................................................................................186

5.2.9 Growth advantage in antibiotics .......................................................................187

5.2.10 Statistics ..........................................................................................................187
5.3 Results ........................................................................................................................................188

5.3.1 Comparison of growth between parent and adapted strains ............................................186

5.3.2 Biofilm growth ..................................................................................................................191

5.3.3 Crystal violet assay of biofilm growth in all strains .........................................................191

5.3.4 Biofilm growth in 2% glucose ..........................................................................................194

5.3.5 Survival in water ..............................................................................................................196

*Escherichia coli* strains .............................................................................................................199

*Staphylococcus aureus* strains ..................................................................................................201

*Pseudomonas aeruginosa* strains ...............................................................................................203

5.4 Discussion ................................................................................................................................205

5.4.1 Fitness cost of antibiotic resistance on ...........................................................................204

Growth ........................................................................................................................................205

Biofilm formation ....................................................................................................................206

Survival in water .......................................................................................................................207

5.4.2 Reduction of biological burden .......................................................................................208

5.4.3 Clinical implications of low-cost resistance .....................................................................211
5.4.4 Growth advantage in antibiotics

6. Chapter six: General discussion and conclusions

6.1 Background

6.2. The role of concentration gradient on the evolution of resistance

6.3. Activated mechanisms of cross-resistance

6.4. Fitness cost of antibiotic resistance

6.5. Conclusion

6.6. Recommendations

6.7. Limitations of study

6.8. Future work

7. References

8. Appendix

8.1 Determination of housekeeping genes

8.1.2. Gene efficiency

8.1.3. norA promoter region
List of Figures

**Figure 1.1**  Diagram of illustrating the difference in cell wall and cell envelope structure of Gram-positive and Gram-negative bacteria..........................39

**Figure 2.1.** Preparation of biocide gradient plates................................................67

**Figure 2.2**  Antibiotic resistant profiles of bacterial strains following biocide priming
........................................................................................................................................77

**Figure 2.3**  Gel electrophoresis and sequence analysis of 16s rRNA gene...................84

**Figure 3.1**  A typical positive ampC β-lactamase result, showing enzymatic inactivation of cefoxitin..........................................................105

**Figure 3.2**  Diagram illustrating the EtBr-agar Cartwheel method............................106

**Figure 3.3**  AmpC β-lactamase test for EcH2O2 and SaCHG.................................112

**Figure 3.4**  The effect of 250 mg/ thioridazine on ciprofloxacin resistance.................118

**Figure 3.5**  The effect of 250 mg/l thioridazine on BKC tolerance............................120

**Figure 4.1**  PCR and sequence analysis of *E. coli* stress response genes.............151

**Figure 4.2**  PCR and sequence analysis of *norA* and *ImrS* genes..........................153

**Figure 4.3**  PCR of *mexJ* and *mexL* genes.................................................................155

**Figure 4.4**  Example of gene efficiency measurement.............................................158
Figure 4.5. Differential expression of *E. coli* stress response genes.................................161

Figure 4.6. Differential expression of *S. aureus norA* and *lmrS* genes...............................163

Figure 4.7. Differential expression of *P. aeruginosa mexJ* and *mexL* genes......................165

Figure 5.1. Comparison of growth between parent and antibiotic resistant strains.

...........................................................................................................................................190

Figure 5.2. Comparison of biofilm formation in parent and biocide adapted strains...193

Figure 5.3. Biofilm growth in 2% glucose............................................................................195

Figure 5.4. Survival in water assay..........................................................................................198

Figure 5.5. Growth advantage of resistant strain EcH2O2 in cephalothin.............200

Figure 5.6. Growth advantage of antibiotic resistant strain SaCHG in oxacillin......202

Figure 5.7. Growth advantage of resistant strain PaGTA in ciprofloxacin............204

Figure 8.1. geNorm validation of housekeeping genes.........................................................312

Figure 8.2. PCR and sequence analysis of *norA* promoter region.................................316

Figure 8.3. Process involved in *ompF* expression...............................................................317

Figure 8.4. Process involved in *norA* expression...............................................................318
List of Tables

**Table 1.1.** Example of biocides and their target sites in bacteria. ........................................27

**Table 1.2.** Example of biocide tolerance and cross-resistance in a variety of bacterial species to biocides and antibiotic. .......................................................................................... 44

**Table 2.1.** Dilution series for hydrogen peroxide. ..................................................................... 58

**Table 2.2** Dilution series of glutaraldehyde .............................................................................. 59

**Table 2.3** Dilution series of chlorhexidine gluconate ................................................................. 60

**Table 2.4** Dilution series of benzalkonium chloride ................................................................. 61

**Table 2.5.** PCR reaction volumes ............................................................................................. 72

**Table 2.6.** MIC of biocides against primed bacteria strains ...................................................... 75

**Table 2.7** Antibiotic susceptibility profiles of pre and post adapted strains ......................... 78

**Table 2.8** MICs of selected antibiotics against parent and primed strains bacteria. 81

**Table 2.9.** Biochemical tests of stable isolates ......................................................................... 82

**Table 3.1.** Examples of major family of efflux pumps and selected antibiotic substrates ................................................................................................................................. 96

**Table 3.2.** Concentrations of efflux pump inhibitor used for each strain ......................... 110

**Table 3.3.** Minimum inhibitory concentrations of efflux pump inhibitors ....................... 114

**Table 3.4.** The effect of TZ on the resistance of EcH2O2 to antibiotics ............................... 115
Table 3.5. The effect of TZ and CPZ on the resistance of SACHG

Table 4.1. PCR primers used in this study

Table 4.2. PCR reaction volume

Table 4.3. Realtime PCR primers used in this study

Table 4.4. Super-mix volumes

Table 4.5. reagents volumes for real time PCR

Table 8.1 Accession numbers of *Escherichia coli* gene primers used in this study

Table 8.2 Accession numbers of *Pseudomonas aeruginosa* gene primers used in this study

Table 8.3 Accession numbers of *Staphylococcus aureus* gene primers used in this study

Table 8.4 Accession numbers of housekeeping gene primers used in this study

Table 8.5. Gene efficiency quantification of primers used in this study
1. Chapter One: General introduction

1.1. Introduction

Biocides are specially formulated compounds employed for the killing and elimination of microorganisms. The use of biocides for both purification and preservation processes and the use of salts for food preservation (Maillard 2002) has been around for hundreds of years, for example, the use of copper and silver vessels for water purification are shown in texts as ancient as the Ayurveda (Davies and Etris 1997; Sudha et al. 2012). The advent of antiseptic surgery by Joseph Lister in the 19th Century introduced the use of disinfectants such as chlorine water and pure phenol, for disease and infection control purposes (Wheeler 1974; Ouyang 2015; Maillard 2002). While disinfectants such as chlorhexidine and quaternary ammonium compounds (QACs) are relatively new, biocides including chlorine-releasing agents and hydrogen peroxide have been in use since the 19th century (Clerck et al. 2007).

Biocides are now used in food and pharmaceutical preparations, preservation, and are applied as disinfectants in healthcare settings to provide clean surfaces (with significantly reduced microbial contamination), equipment and materials in order to prevent and reduce contamination. Biocides have also found their way into households in cleaning and personal care products such as toothpastes, surface sprays, hand wash
and washing powders (Gnanadhas et al. 2013; Kurenbach et al. 2015; Wales and Davies 2015).

The potency and actions of different types of biocides against microorganisms varies, depending on the type of microorganisms and strains (even within the same species). It is therefore necessary to apply them according to microbial susceptibility levels (Mangram et al. 1999; Maillard 2002; Liu et al. 2015; Webber et al. 2015; Tronsmo et al. 2016). Ranking of microorganisms according to their biocidal susceptibility levels has improved the knowledge of factors involved in their antimicrobial activities, for example, *Mycobacteria species* are less susceptible to biocides due to their cell wall (Trias and Benz 1994), compared to Gram-negative bacteria, with Gram-positive bacteria ranking most susceptible (Maillard 2002; Otter et al. 2015). Gram-positive bacteria are more sensitive in comparison to Gram-negative bacteria because their cell walls are made up of peptidoglycan and teichoic acid which, unlike lipopolysaccharide (LPS) in Gram-negative bacteria, does not prevent entry of biocides (Denyer and Maillard 2002).

Gram-negative bacteria are less susceptible to biocides due to their complex double outer membrane (OM) (Stavenger and Winterhalter 2014; Zgurskaya et al. 2015; Silver 2016; Masi and Pages 2019). The double cell membrane consists of an outer lipopolysaccharide (LPS) layer (which contains both lipid A and O antigen chains) and a phospholipid inner leaflet (Nikaido 2003; Masi and Pages 2019). It can reduce the permeability of antimicrobials and negatively affect their critical intracellular concentrations (Hugo et al. 1999; Maillard 2002; Moore and Payne 2004; Phetsang et al.
2016; Masi and Pages 2017). Although Gram-positive bacteria are generally more sensitive to biocides, they can sometimes become less sensitive depending on their physiological state, for example, nutrient depletion can cause increased peptidoglycan thickening and crosslinking which can affect the amount of substances that can pass through the cell wall (Liaqat and Sabri 2008; Russell 2003a). An example of the effect of physiological state on change in antimicrobial susceptibility of Gram-positive bacteria was described by Kolawole in a 1984 experiment where strains of S. aureus became more sensitive to chlorhexidine (CHG) when their surrounding mucoid layers were removed, indicating that the mucoid layer was a protective barrier against the biocide (Kolawole 1984).

These outer membranes are however spanned by water-filled β-barrel channels (porins) that allow the diffusion of amphiphilic compounds (Schulz 2002; Nikaido 2003; Delcour 2003; Masi and Pages 2019). The outer membrane therefore acts as first line of defence against noxious compounds by reducing their intracellular concentrations and in turn contributing to their tolerance (Nikaido and Vaara 1985; Gilbert et al. 1990a; Nikaido 2003; Masi and Pages 2019). There are three well studied porins in E. coli; OmpF, OmpC and PhoE (Pagès et al. 2008; Masi and Pages 2019). These porins are known for their proclivity for charge and size, both OmpF and OmpC prefers cationic to anionic compounds compared to PhoE which prefers anions but OmpF prefers larger solutes compared to OmpC (Masi and Pages 2019). The reduced expression of porin genes such as oprD in P. aeruginosa have also been shown to contribute to increase tolerance in the bacteria (Büscher et al. 1987; Godfrey and Bryan 1987).
1.2 Selected biocides and their mode of action

Examples of biocides classified based on their target sites on microorganisms are shown in Table 1.1. The biocides used in this present study are; hydrogen peroxide, chlorhexidine, benzalkonium chloride and glutaraldehyde. Their mechanisms and modes of action will be discussed in this section.
Table 1.1. Example of biocides and their target sites in bacteria

<table>
<thead>
<tr>
<th>Biocide</th>
<th>Target in bacteria</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Quaternary ammonium compounds</td>
<td>Cell membrane</td>
<td>(Ioannou et al. 2007)</td>
</tr>
<tr>
<td>Isothiazones</td>
<td>Cell membrane</td>
<td>(Patel and Desai 2014)</td>
</tr>
<tr>
<td>Biguanides</td>
<td>Cytoplasmic membrane</td>
<td>(McDonnell and Russell 1999)</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Proteins</td>
<td>(McDonnell and Russell 1999)</td>
</tr>
<tr>
<td>Heavy-metal derivatives</td>
<td>Proteins</td>
<td>(McDonnell and Russell 1999)</td>
</tr>
<tr>
<td>Alcohols</td>
<td>Nucleic acids</td>
<td>(McDonnell and Russell 1999)</td>
</tr>
<tr>
<td>Peroxygens</td>
<td>Nucleic acids, proteins and lipids</td>
<td>(Block 2001)</td>
</tr>
<tr>
<td>Halogen releasing agents</td>
<td>Proteins</td>
<td>(Bloomfield 1996)</td>
</tr>
<tr>
<td>Phenols</td>
<td>Cell membrane</td>
<td>(Denyer 1995)</td>
</tr>
<tr>
<td>Chlorine releasing compounds</td>
<td>Cell wall</td>
<td>(McDonnell and Russell 1999)</td>
</tr>
<tr>
<td>Bisphenols</td>
<td>Cytoplasmic membrane</td>
<td>(Regos and Hnz 1974)</td>
</tr>
</tbody>
</table>
1.2.1. Hydrogen peroxide

Hydrogen peroxide (H$_2$O$_2$) was first discovered in 1818 by Louis Jacques Thenard (Maehly and Chance 1954) and is a colourless, safe to use, broad-spectrum biocide. H$_2$O$_2$ is useful in both health and industrial applications against bacteria, yeasts, viruses and bacterial spores (McDonnell and Russell 1999; Block 2001; Linley et al. 2012). The property of H$_2$O$_2$ to rapidly degrade into water and oxygen makes it an environmentally friendly biocide of choice (McDonnell and Russell 1999; Linley et al. 2012). Increase tolerance to H$_2$O$_2$ due to catalases and peroxidases have previously been reported in Gram-negative and Gram-positive bacteria such as *E. coli* and *S. aureus* (Imlay and Linn 1987; Bogomolnaya et al. 2013; Painter et al. 2015). Catalases are protective enzymes produced by cells to prevent oxidative damage brought about by reactive oxygen species, achieved by decomposing hydrogen peroxide to oxygen and two molecules of water (2H$_2$O$_2$ → 2H$_2$O + O$_2$) (Alfonso-Prieto et al. 2009) and peroxidases are known for their ability to catalyse the dehydrogenation of organic compounds like phenols and aromatic amine (Pütter 1974).

Two modes of killing have previously been described of H$_2$O$_2$ against *E. coli* (Imlay and Linn 1986; Imlay and Linn 1987). *Escherichia coli* strains lacking RecA proteins (essential for DNA repair and maintenance), DNA polymerase I, exonuclease III and exonuclease V (required for DNA repair) have been shown to be more prone to mode one killing, suggesting that DNA is the target at low concentration of H$_2$O$_2$ (Imlay and Linn 1988; Imlay and Linn 1987). Mode one killing is activated at H$_2$O$_2$ concentrations of 1 to 2 mM (Imlay and Linn 1986), in the presence of metabolically active cells during H$_2$O$_2$ exposure.
and usually leads to DNA damage. While mode two happens in the absence of metabolism and at higher concentrations of H$_2$O$_2$ (>10 mM) after prolonged exposure due to damage to all macromolecules (Imlay and Linn 1986; Imlay and Linn 1987; Brandi et al. 1989; Uhl et al. 2015). Exposure to low concentrations of H$_2$O$_2$ can activate a protective response from E. coli, promoting increased tolerance during subsequent exposure (Demple and Halbrook 1983; Christman et al. 1985). Two non-overlapping protective stress responses are activated during E. coli exposure to H$_2$O$_2$; the oxyR regulon which upregulates the production of scavengers of reactive oxygen species and the SOS response (a global cell cycle arrest in response to DNA damage) (McKenzie et al. 2000), which is important in recombinant DNA repair due to ROS damage (Imlay and Linn 1986, Imlay and Linn 1987; Hidalgo and Demple 1996; Pomposiello and Demple 2001; Chiang and Schellhorn 2012).

Hydrogen peroxide also activates the soxRS regulon, a protective stress response system found in E. coli (Manchado et al. 2000), this is in contrast to previously accepted findings of superoxide being the only activator of this regulon (Touati 1988; Greenberg and Demple 1989; Walkup and Kogoma 1989; Nunoshiba et al. 1992; Manchado et al. 2000). The soxRS regulon is a member of the merR family of transcriptional regulators and controls the activation of the soxRS operon (Wu and Weiss 1991; Nunoshiba et al. 1992). Oxidation of the two 2Fe-2S clusters in soxRS is required for the activation of soxR, resulting in a structural change which in turn signals the transcription of soxS (Hidalgo and Demple 1994; Hidalgo et al. 1995; Ha et al. 2005). Once translated, the SoxS protein controls the expression of over 100 oxidative stress response genes (Blanchard et al.
2007), such as *acrAB* and *micF* (which in turn negatively regulates *ompF* expression). According to previous studies, when the activating stress condition is no longer present both SoxR and SoxS are removed, SoxR is reduced by the gene products rsxBC and rseC allowing SoxS to be degraded (Koo *et al.* 2003; Griffith *et al.* 2004).

Generation of reactive oxygen species such as $O_2^-$ and $H_2O_2$ is important in host defence system to remove bacteria such as *S. aureus* (Liu *et al.* 2005; Liu *et al.* 2008). *Staphylococcus aureus* protects itself by launching a host of protective molecules such as catalase and superoxide dismutase (Valderas and 2001; Liu *et al.* 2005). Oxidative stress brought about by low concentrations of $H_2O_2$ have previously been reported to cause antibiotic resistance in Gram-positive bacteria such as *S. aureus* (Painter *et al.* 2015). Low concentrations of the biocide have previously been shown to introduce mutation in *S. aureus* via double-strand DNA break and repair mechanism, which in turn causes increased catalase production leading to tolerance to $H_2O_2$ and increased resistance to antibiotics such as gentamicin, an aminoglycoside which inhibits protein synthesis in some bacterial strains (Painter *et al.* 2015).

There is evidence that catalase producing *P. aeruginosa* can prevent the penetration of $H_2O_2$ especially in biofilm producing strains (Hassett *et al.* 1999; Ma *et al.* 1999; Stewart *et al.* 2000). This was demonstrated in the work of Stewart *et al.*, where they showed reduced penetration of the biocide in *katA* and *katB/katA* mutant *P. aeruginosa* biofilms compared to wild-type *P. aeruginosa* with intact *katA* and *katB* genes (Stewart *et al.* 2000).
1.2.2. Chlorhexidine

Chlorhexidine (CHG) is a cationic bisbiguanide (Davies et al. 1954; Davies 1973) that inactivates microorganisms by crosslinking adjacent anionic phospholipid head groups present on their cell surfaces (Chawner and Gilbert 1989). Crosslinking of acidic phospholipid head groups removes their attached cations (Mg$^{2+}$ and Ca$^{2+}$ (Davies 1973) and disrupts membrane fluidity (Hugo and Longworth 1966). The biocide is applied as a preservative in pharmaceutical and cosmetic products and as a broad-spectrum antiseptic (Curd and Rose 1946; Rose and Swain 1956; Hardy et al. 2017). The action of CHG on bacteria can either be bacteriostatic (to impair) or bactericidal (to kill)(Davies 1973), depending on the concentration employed. When used at higher concentrations (200-500 mg/l) the effect is the precipitation and coagulation of the cytoplasmic content (Hugo and Longworth 1965; Chawner and Gilbert 1989; Maillard 2002) and at low concentrations (90 mg/l ) it causes cytological damage and the loss or release of cell contents (Hugo and Longworth 1965).

1.2.3. Benzalkonium chloride

Benzalkonium chloride, a quaternary ammonium compound (QAC) is a cationic biocide that inactivates bacteria by interacting with and disrupting their cell membranes (Gilbert and Moore 2005). Activation of multidrug efflux systems such as the resistance-nodulation-cell division (RND)-type MexCD-OprJ in Pseudomonas species has been linked to the use of sub-inhibitory concentrations of membrane damaging biocides such as BKC and CHG (Morita et al. 2003; Fraud et al. 2008). As was shown in the work of
Loughlin et al. where adaptation of *Pseudomonas aeruginosa* to sub-inhibitory concentrations of BKC was attributed to increased efflux (Loughlin et al. 2002; McCay et al. 2010).

1.2.4. Glutaraldehyde

Glutaraldehyde (GTA) was first synthesized in 1908 (Russell 1994; Cholley et al. 2020) and is a derivative of mono or polymeric aldehydes (Russell 1994). GTA is a biocide that is employed in the prevention and control of microbial and biofilm growth (Russell 1994; Russell 2002c; Maillard 2007; Meyer and Cookson 2010; McDonnell and Burke 2011). The use of aldehydes such as GTA as a disinfectant is no longer recommended in the United Kingdom and France due to their fixative properties (Beilenhoff et al. 2018). Glutaraldehyde is effective in both mechanical and automated endoscope disinfection due to its ability to penetrate and erode microbial biofilms while inhibiting microbial growth (Russell 1994; Tschudin-Sutter et al. 2011; Beilenhoff et al. 2018; Cholley et al. 2020). The biocide acts as an inhibitor of protein synthesis (Cholley et al. 2020) and as an alkylating agent by attaching to free amino groups present on the surfaces of microorganisms to create cross-linked proteins (McDonnell and Russell 1999; Russell 2002c; Cholley et al. 2020). This action leads to both disruption and depolarisation of cell and cytoplasmic membranes and subsequent leakage of cytosolic components (Al-Adham et al. 1998; Simoes et al. 2006).
1.3. Bacterial tolerance to biocides

Antibiotic resistance is a global public health problem, the major contributor being the suboptimal use and misuse of antibiotics in both human and veterinary medicine (Gnanadhas et al. 2013). While antibiotics have specific target sites in bacteria (Chopra et al. 2002), biocides have multiple target sites and are therefore less specific and their effect are mostly fast and lethal rather than inhibitory (Maillard 2002; Gilbert and McBain 2003; Morente et al. 2013).

The association between reduced susceptibility of clinical isolates of bacteria to biocides (Dance et al. 1987; Griffiths et al. 1997; Fraise 2002; SCENIHR 2010; Vikram et al.; 2015 Knapp 2015; Wand et al. 2016) with subsequent high occurrence of antimicrobial resistance (Stickler and Thomas 1980) has led to growing concerns within the scientific community. The concern is that the use of biocides and preservatives in clinical, domestic and industrial settings may have contributory effects on the development and selection of antibiotic resistant strains (Stickler and Thomas 1980; Mycock 1985; Sasatsu et al. 1992; Russell et al. 1998; Levy 1998a; Levy 1998b; Murtough et al. 2000 Russell 2002b).

Resistance to biocides are not as common as resistance to antibiotics, but there have been several reports of bacterial resistance to biocides such GTA (Kirschke et al. 2003; Simoes et al. 2006; Svetlikova et al. 2009; Simões et al. 2011; Tschudin-Sutter et al. 2011). Svetlikova et al showed that the increased resistance to GTA in *Mycobacterium smegmatis* was due to a mutation in the *mspA* porin, which was observed to cause
reduced binding of and penetration of the biocide (Svetlikova et al. 2009). Another study using _P. fluorescens_ biofilm shows that exposure of the bacteria to GTA significantly induced two multidrug efflux genes, PFLU2929 and PFLU3876 which appear to be orthologs of the oprN and the PA5159 both of which encodes components a multidrug transporter in _P. aeruginosa_ strain _PAO1_ (Vikram et al. 2015).

To learn how biocides may contribute to biocide tolerance (increased bacterial resilience) and antibiotic cross-resistance in bacteria, it is important to understand the mechanisms involved, conditions that contribute to bacterial tolerance mechanisms are discussed below.

1.3.1. Biofilms

The attachment, proliferation and colonisation of a community of microorganisms to surfaces usually results in biofilm formation (Hall-Stoodley et al. 2004; Tuson and Weibel 2013; Pavithra and Doble 2008). While their initial attachment may be reversible, it is the secretion of exopolysaccharide by attached bacteria that enhances their adherence (Wu et al. 2018). Formation of biofilms may allow bacteria to withstand physical and chemical stress conditions (Brown and Gilbert 1993; Gilbert et al. 2002; Norouzi et al. 2010). Previous studies show that biofilm formation contributes to tolerance of _Staphylococcus epidermidis_ and _Pseudomonas aeruginosa_ to tobramycin and benzalkonium chloride (Nickel et al. 1985; Evans and Holmes 1987; Gristina et al. 1987; Prosser et al. 1987; Gilbert et al. 1990b). A study by Marrie and Costerton in 1981 showed that strains of _Serratia marcescens_ that were isolated from the walls (embedded
in a fibrous matrix) of plastic bottles containing 2% chlorhexidine, which were originally susceptible to 1024 ug/ml of the biocide were subsequently able grow in 20,000 ug/l (2%) concentration of chlorhexidine (Marrie and Costerton 1981). Other studies found that formation of biofilms may also contribute to the resistance of some bacterial strains to biocides (Joseph et al. 2001). For example, Wong et al in their 2010 study observed the survival of a 3-day-old *Salmonella enterica* serovar Typhimurium biofilm after it was exposed to in use concentrations of quaternary ammonium compounds, sodium hypochlorite, citric acid, ethanol and benzalkonium chloride respectively (Wong et al. 2010). Their study indicates that using recommended concentrations and contact times for biocides reduces microbial survival.

1.3.2. Upregulation of efflux pumps

The intrinsic resistance of Gram-negative bacteria to antimicrobials was previously ascribed to the diffusion barrier of their outer membrane alone (Nikaido 1989). Studies on *P. aeruginosa* however found that the majority of antimicrobial compounds could pass through its strong outer membrane in less than one minute, indicating that there may be other mechanisms contributing to its resistance (Li et al. 1994; Nikaido 1989). Efflux pumps are major contributors to biocidal resistance mechanisms in Gram-negative bacteria (Nikaido 1998; Poole 2001; Li and Nikaido 2004; Poole 2004). The acridine resistance proteins, AcrAB, encoded efflux pump is responsible for multi-drug resistance in Gram-negative bacteria (George and Levy 1983). The presence of the AcrAB pump in *E. coli* allows it to pump out various compounds such as ethidium bromide,
phenylethylalcohol and sodium dodecyl sulphate (Ma et al. 1993; Ma et al. 1994). Other efflux pumps, such as the multidrug resistance proteins MexAB, MexCD and MexEF encoded by \textit{P. aeruginosa}, are responsible for pumping out antimicrobials such as triclosan using proton motive force (Schweizer 1998; Poole 2001).

Although resistance due to efflux pumps is more common amongst Gram-negative bacteria, some Gram-positive bacteria are also known to possess efflux pumps, for example \textit{S. aureus} encodes efflux pump genes \textit{qacA} to \textit{qacH}, responsible for its resistance to ethidium bromide, acriflavine and quaternary ammonium-type antiseptic compounds (Rouch et al. 1990). The QacG, QacH and QacJ pumps have been shown to increase the tolerance of \textit{Staphylococcus haemolyticus} to biocides such as QAC and biguanides (Correa et al. 2008). The QuacA, QuacB and SmR efflux pumps were shown to increase chlorhexidine gluconate tolerance in methicillin-resistant \textit{S. aureus} (MRSA) isolates from two intensive care units in Canada (Longtin et al. 2011).

To confirm the involvement of efflux pumps in bacterial intrinsic resistance, Mavri \textit{et al.} in their 2012 experiments used phenylalanine-arginine \(\beta\)-naphthylamide and 1-(1-naphthylmethyl)-piperazine (putative efflux blockers) to reverse resistance of \textit{Campylobacter jejuni} and \textit{Campylobacter coli} to biocides such as triclosan, benzalkonium chloride and chlorhexidine diacetate and antibiotics Erythromycin and Ciprofloxacin (Mavri and Možina 2012).
1.3.3. Degradation and inactivation of biocides

Bacteria such as *Pseudomonas putida*, *Pseudomonas fluorescens* and *Achromobacter xylosoxidans* have been shown to inactivate or degrade biocides. *Pseudomonas species* are known to inactivate biocides including triclosan, didecyldimethylammonium chloride (a QAC) and phenols, while *A. xylosoxidans* can degrade chlorhexidine (Ogase *et al.* 1992; Vanklingerern and Pullen 1993; Nishihara *et al.* 2000; Meade *et al.* 2001). A 1992 study carried out by Ogase *et al.* showed that two chlorhexidine resistant strains of *A. xylosoxidans* isolated from ultrasonic hand washer could degrade chlorhexidine when decomposition products; phenol and p-chlorophenol were detected by three dimensional HPLC after the strains were exposed to the biocide (Ogase *et al.* 1992).

1.3.4. Modification of target sites

Resistance due to target site mutations are not common in biocide resistant bacteria, due to their less specific target sites, in comparison with the highly specific targets of antibiotics (Poole 2002), however there are some exceptions, such as triclosan resistance in *E. coli*. The target site for triclosan 5-chloro-2-(2,4-dichlorophenoxy) phenol is the *fabI* gene (enoyl-acyl carrier protein reductase), which is important in fatty acid synthesis (Heath *et al.* 1998). Crystallography studies shows interaction between triclosan and the FabI, whereby it was shown to form a stable ternary complex with triclosan and NAD⁺ through hydrogen bonds and hydrophobic interactions (Heath *et al.* 1999). The presence of triclosan increases the affinity of FabI for NAD⁺, with triclosan binding at the enoyl substrate site to inhibit essential fatty acid synthesis (Heath *et al.*
Missense mutation in the FabI active site prevents the FabI affinity for NAD⁺, preventing the FabI-NAD⁺-triclosan complex (Heath et al. 1999).

1.3.5. Impermeability

Most bacteria apart from L-forms and Mycoplasma (Dienes and Bullivant 1968; Lambert 2002) possess rigid cell walls, which are important in their growth and protection in hostile environments. The cell wall controls bacterial cell shape supports cytoplasmic content and acts as a barrier against noxious compounds, Figure 1.1 compares the cell wall components of Gram positive and Gran-negative bacteria (Lambert 2002). The function of the cell structure as a protective barrier against noxious compounds varies in Gram negative, Gram-positive and Mycobacteria (Lambert 2002; Seltmann and Holst 2013). Gram-positive bacteria possess a permeable cell wall which allows unrestricted penetration of antimicrobials (Koch 2000; Lambert 2002). However, resistance due to reduced permeability can occur as was previously described in vancomycin resistant S. aureus (Cu et al. 2000), where increased amounts of non-amidated glutamine residues in the peptidoglycan caused increased cell wall thickening that led to vancomycin trapping (Cu et al. 2000). Compared to the permeable cell wall of Gram-positive bacteria, Mycobacteria cell wall, with its high in lipid content prevents the penetration of hydrophilic antimicrobials but allow hydrophobic ones like rifampicin and fluoroquinolones to enter via its hydrophobic bilayer which contains long chains of mycolic acid and glycolipids (Christensen et al. 1999; Lambert 2002). The presence of
porins in the lipid bilayer of *Mycobacteria*, for example the MspA porins used for nutrient transport (D’Arcy and Taylor 1962), suggests similarity to the outer cell.

**Figure 1.1. Diagram of illustrating the difference in cell wall and cell envelope structure of Gram-positive and Gram-negative bacteria.** Adapted from Green 2002.

Cell wall structure of (a) Gram-positive bacteria cell and (b) Gram-negative bacteria. The cell walls of both Gram-positive and Gram-negative bacteria are made up of phospholipid and protein.

While both cell walls contain peptidoglycan, it is however more abundant in the Gram-positive, with intercalating lipoteichoic acid.

In the Gram-negative bacteria wall, the peptidoglycan is located between the outer and inner membranes, the Gram-negative outer cell membrane is largely made up of phospholipids, lipopolysaccharide (LPS) and porins which selectively regulates the flow of molecules across (Green 2002).
membrane of Gram-negative bacteria (Lambert 2000; Masi and Pages 2019). Studies show that Gram-negative bacteria can modify their outer membranes to prevent biocide uptake, for example phenol resistance in *E. coli* may be due to its ability to modify outer membrane proteins (Zhang *et al.* 2011).

In Gram-negative bacteria, lipopolysaccharides (LPS) are a major component of the outer membrane. Studies show that changes in the expression of LPS in *S. typhimurium* and *P. aeruginosa* increased the sensitivity of these bacteria to antimicrobials (Russell and Furr 1986; Russell and Furr 1987; Russell 1995), highlighting the significance of LPS in antimicrobial resistance. Other studies observed that stepwise training of Gram-negative bacteria to cationic biocides may modify their LPS and therefore increase their tolerance to subsequent exposure (Cox and Wilkinson 1991), as was seen in *P. aeruginosa* after exposure to QACs (Guérin-Méchin *et al.* 1999; Guerin-Mechin *et al.* 2000) where changes in the composition of their outer membranes after exposure to QACs corresponded to their increased tolerance to higher concentrations of the biocides.

1.4. Evidence of biocide-antibiotic cross-resistance

Continuous exposure of bacteria to sub-inhibitory concentrations of biocides may lead to reduced susceptibility as well as cross-resistance to other biocides and antibiotics (Gilbert and McBain 2003). For example, elevation in the minimum inhibitory concentration (MIC) of 2-phenoxyethanol and cross-resistance to other biocides such as chlorhexidine and benzisothiazolone was observed when *P. aeruginosa* was treated with
2-phenoxyethanol, however no cross-resistance with antibiotics was observed (Malek and Badran 2010).

Chuanchuen et al. in their 2001 experiments observed an increased expression of the mexCD-oprJ efflux system, when susceptible *P. aeruginosa* were exposed to triclosan. This exposure caused up to 500-fold increase in MIC and reduced susceptibility to several drugs; including a 94-fold increased MIC to ciprofloxacin due to mutation in the *nfxB* (the mex-operon regulatory gene) (Chuanchuen et al. 2001).

Exposure of *Stenotrophomonas maltophilia* to triclosan induced a phenotypic antibiotic resistance to quinolones, binding of triclosan to *smeT*, (a repressor of the multidrug efflux pump *smeDEF*) releases it from its promoter allowing for the expression of *smeDEF* and subsequent increased tolerance of *S. maltophilia* to quinolone antibiotics such ciprofloxacin and nalidixic acid (Hernández et al. 2011). In their 2012 experiments, Soumet et al. (2012) observed reduced antibiotic susceptibility in *E. coli* (from 3.5 to 7.5-fold) to phenolic compounds (β-lactams, and quinolones) and resistance to florfenicol and chloramphenicol, after a seven-day exposure to increasing sub-inhibitory concentrations of quaternary ammonium compounds. This was attributed to enhanced efflux expression (Langsrud *et al*. 2004; Soumet *et al*. 2012). A 2014 study by Capita *et al* found that *E. coli ATCC 12808* developed stable increased tolerance to aminoglycosides, cephalosporins and quinolones after exposure to sub-lethal concentrations of trisodium phosphate, sodium nitrite and sodium hypochlorite (Capita *et al*. 2014).
In a 2015 study by Kurenbach et al, exposure of E. coli to sub-inhibitory concentrations of the herbicides dicamba and glyphosate reduced the susceptibility of the bacteria to chloramphenicol and kanamycin respectively. The role of SoxS as an inducer of the AcrAB efflux pump was also confirmed (Kurenbach et al. 2015). Braoudaki et al observed the ability of benzalkonium chloride-resistant Salmonella enterica serovar Virchow to develop elevated resistance to chlorhexidine while chlorhexidine-resistant strain of the same bacteria failed to develop cross-resistance to benzalkonium chloride. These findings suggest that resistance and cross-resistance mechanisms may be strain specific and not generic (Braoudaki and Hilton 2004a; Braoudaki and Hilton 2004b).

In their 2010 work, McCay et al observed cross-resistance with ciprofloxacin in their BKC primed P. aeruginosa grown in continuous culture (McCay et al. 2010), this contributes to the findings of Loughlin et al where cross-resistance was observed with other QACs after serial batch culture (Loughlin et al. 2002). The varied outcome in these studies shows how different growth conditions may influence adaptation and selection of bacteria to antimicrobials.

A study by Wand et al showed an increased tolerance of Klebsiella pneumoniae to chlorhexidine after exposure to sub-lethal concentrations of the biocide. The same group has also showed a link between increased tolerance of K. pneumoniae to chlorhexidine with cross-resistance to the antibiotic colistin (Wand et al. 2016). Genome sequencing analysis of both parent and tolerant strains indicated that mutations in the phoPQ (a 2-component regulator) and smvR (a putative tetracycline repressor) genes
were responsible for the cross-resistance between chlorhexidine tolerance and colistin resistance (Wand et al. 2016, Bock et al. 2016). Example of biocide tolerance and antibiotic cross-resistance in variety of species are shown in Table 1.2.
Table 1.2. Example of biocide tolerance and cross-resistance in a variety of bacterial species to biocides and antibiotic.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Biocide tolerance</th>
<th>cross-resistance</th>
<th>Mechanisms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>Triclosan</td>
<td>Ciprofloxacin</td>
<td><em>nfxB</em> gene mutation</td>
<td>(Chuanchuen <em>et al.</em> 2001)</td>
</tr>
<tr>
<td><em>Salmonella enterica serovar Typhimurium</em></td>
<td>Triclosan</td>
<td>Chloramphenicol</td>
<td>Efflux system</td>
<td>(Birošová and Mikulášová 2009)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>BKC and dioctyl dimethyl ammonium chloride.</td>
<td>Ceftazidime &amp; Cefoxim.</td>
<td>Efflux system</td>
<td>(Srinivasan <em>et al.</em> 2014)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Triclosan</td>
<td>Ciprofloxacin</td>
<td>Cell membrane</td>
<td>(Tkachenko <em>et al.</em> 2007)</td>
</tr>
<tr>
<td><em>M. chelonae</em></td>
<td>Glutaraldehyde</td>
<td>Rifampicin and Ethambutol</td>
<td>Altered cell wall Polysaccharides</td>
<td>(Manzoor <em>et al.</em> 1999)</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Chlorhexidine</td>
<td>Colistin</td>
<td><em>phoPQ and smvR</em></td>
<td>(Wand <em>et al.</em> 2016)</td>
</tr>
</tbody>
</table>
1.5. Gap in current knowledge

Antimicrobial resistance is one of the greatest problems facing mankind. Development of resistance is so far spread that it has now become a global challenge (Woodford and Livermore 2009; Piddock 2012; Bock et al. 2016; Amos et al. 2018). In the conclusion of their first global report on antimicrobial resistance, the World Health Organisation (WHO) stated that the World may be heading back to an era where minor infections and injuries will become fatal once again, unless there is quick intervention (World Health Organization 2014). According to the first economic report on the impact of antimicrobial resistance published in 2014, by 2050 there may be a rise from 700,000 to 10 million in annual deaths relating to antibiotic resistance if nothing changes in the fight against it (O’Neill 2014). Most traditional antimicrobials are now failing in the fight against resistance (Walsh and Toleman 2011; Roca Subirà et al. 2012; Tuon et al. 2012).

Biocides have been used for centuries to control infectious agents (Russell 2002b; Maillard 2002) and they are either applied as or added to several formulated products that are used as disinfectants, preservatives, pesticides, antiseptics and cosmetics, for example hand creams and soaps (Maillard 2002; Knapp et al. 2015). Public awareness to infection control has caused a rise in the use of biocide and biocidal products in the home environment (Fraise 2002; Knapp et al. 2015). As a result of this uncontrolled use of biocides (especially at sub-inhibitory concentrations), there is now growing concerns over how their selective pressure may potentially favour the development of less susceptible bacteria strains as well as encourage the expression and dissemination of resistance mechanisms (Fraise 2002, SCENIHR 2010, Maillard, Jean-Yves et al. 2013;
A major concern is the effect of sub-inhibitory concentrations of biocides on the increased tolerance or resistance of bacteria (SCENIHR 2010). Several laboratory studies have demonstrated a link between exposure of bacteria to sub-inhibitory concentrations of biocides such as trichlorocarbanalide (TCC), didecyldimethylammonium chloride (DDDMAC) and C10-16-alkyldimethyl, N-oxides, (ADMAO) increased tolerance and resistance to antibiotics such as chloramphenicol (Walsh et al. 2003; Escalada et al. 2005; Christensen et al. 2011; Knapp et al. 2013; Bock et al. 2016).

Other studies have demonstrated antibiotic cross-resistance in bacteria after exposure to sub-inhibitory concentrations of biocides (Kurenbach et al. 2015; Wand et al. 2016). Since biocides have several target sites within bacteria (Maillard 2002; Russell 2002b), the most common mechanisms for cross-resistance are via non-specific processes such as efflux-pumps (Costa et al. 2013; Bogomolnaya et al. 2013).

A 2013 review by Maillard et al and a report from The Scientific Committee for Emerging and Newly Identified Health Risks (SCENIHR) identified biocides as a risk to resistance due to selective pressure for less susceptible strains. Their findings highlighted a key gap in current knowledge in understanding the effect of low concentrations of biocides on bacterial cells, as well as the mechanisms involved in the development of resistance and cross-resistance (Maillard 2002; Jaffe et al. 1982; SCENIHR 2010; Knapp et al. 2015). Due to these concerns, the European Union and The United States have proposed changes in the regulations, requiring manufacturers of biocidal products to provide data on the
risks of development of tolerance in organisms targeted by biocidal products (SCENIHR 2010; Knapp et al. 2015).

In previous studies, the effect of biocide on antibiotic-cross resistance have only been looked at after increased tolerance or resistance to biocides were observed in bacteria (Slayden et al. 2000; Chuanchuen et al. 2001; Braoudaki and Hilton 2004a; Tkachenko et al. 2007; Soumet et al. 2012; Mavri and Možina 2012; Wand et al. 2016).

1.6. Aims and objectives of this study

The aims and objectives of this study are:

- To understand the effects of sub-inhibitory concentrations of biocides priming and bacterial resistance, this will be achieved by exposing them to increasing concentrations of biocide following gradient plate and stepwise methods of mutant generation.

- To identify increased tolerance to biocides and cross-resistance to the other biocides and antibiotics, this will be achieved using EUCAST methods to measure minimum inhibitory concentrations (MIC) and disc diffusion tests.

- To assess the presence of active antibiotic resistance mechanisms such as efflux pumps and β-lactamase enzymes. This will be done by testing the effects of efflux pump inhibitors on the susceptibility of biocide adapted strains of bacteria and using ethidium bromide cartwheel method to compare the efflux of ethidium bromide from antibiotic resistant and biocide tolerant cells in comparison to wild type.
To test the effect of β-lactamase by using Ethylene-diamine tetra acetic acid (EDTA)/saline solution to permeabilise antibiotic resistant bacterial cells in order to release β-lactamase enzyme into the surrounding media in comparison to wild-type strains. This will be measured by comparing the zone of inhibition around filter discs impregnated with EDTA to the antibiotic disc used.

To identify the molecular mechanisms involved in both increased tolerance and cross-resistance with antibiotics, this will be achieved by comparing the expression of known antimicrobial resistance genes in parent and biocide primed strains of bacteria using both PCR and quantitative real time PCR.

To see if any observed biocide tolerance and antibiotic resistance comes with fitness costs (deficiencies, e.g. reduced growth resulting from increased antibiotic resistance). This will be carried out by comparing growth rate, biofilm formation and survival in water between biocide primed and wild type strains.
2. Chapter Two: priming with biocides: a pathway to antibiotic resistance

2.1. Introduction

Horizontal gene transfer, changes in composition of outer membranes and selective pressure exerted by antimicrobials are the main contributors to antibiotic resistance (Hooper 2000; 2001; Hawkey 2003). Mutations in gene targets due to exposure to high concentration of antimicrobials are the usual methods of studying antibiotic resistance in bacteria (Piddock 1999; Li and Nikaido 2004; Levy and Marshall 2004), but studies have shown that constant exposure of bacteria to sub-inhibitory concentrations of antimicrobials can allow for selection of tolerant or resistant strains (Manzoor et al. 1999; Chuanchuen et al. 2001; Vali et al. 2008; Wand et al. 2016). Compared to exposure to lethal concentrations of antimicrobials, where there is survival of the fittest and highly resistant strains, prolonged exposure to low concentrations allows for a much-extended window of time for selective pressure to be exerted. At sub-inhibitory concentrations for extended periods, the chance of developing reduced susceptibility to antimicrobials may be higher compared to when exposed to lethal concentrations for short periods (Gullberg et al. 2011; Hughes and Andersson 2012; Andersson and Hughes 2014).

As discussed in section 1.1, biocide-targets in bacteria are less specific and therefore resistance to biocides is less common (Gnanadhas et al. 2013). There are however several instances where reduced tolerance to biocides have emerged due to
mechanisms such as increased efflux which also contributes to bacterial survival (Slayden et al. 2000; Chuanchuen et al. 2001; Braoudaki and Hilton 2004a; Tkachenko et al. 2007; Soumet et al. 2012; Mavri and Možina 2012; Wand et al. 2016).

As previously demonstrated by several studies and discussed in length in section 1.4, prolonged exposure of bacteria to biocides can generate less susceptible strains which can often display reduced susceptibility to various antibiotics (Kimon et al. 2007; Randall et al. 2007; Karatzas et al. 2008; Whitehead et al. 2011; Karatzas; Fernández et al. 2017; Hardy et al. 2017). Environmental contamination with antibiotics has already resulted in the selection for antibiotic-resistant strains of bacteria present in food crops and water surfaces (Gaze et al. 2011; Ashbolt et al. 2013; Wellington et al. 2013). There are reports of common biocides such as triclosan and quaternary ammonium compounds being transferred to the environment via human urine and breast milk (Adolfsson-Erici et al. 2002; Li et al. 2013; Savage et al. 2014; Zhang et al. 2015; Halden et al. 2017), it would be interesting to know if the prolonged presence of these biocides in the environment also exert selective pressure as with previous laboratory studies (Gullberg et al. 2011; Hughes and Andersson 2012; Andersson and Hughes 2014).

While exposure to low concentrations of biocides has been linked to reduced susceptibility and cross-resistance to antibiotics (Slayden et al. 2000; Chuanchuen et al. 2001; Braoudaki 2004a; Soumet; Tkachenko et al. 2007; Fourreau et al. 2012; Mavri, Možina 2012; Wand et al. 2016), this has often been with corresponding increased tolerance to biocides, it would therefore be interesting to see the effect of low
concentrations of biocides on antibiotic resistance in the absence of increased biocidal
tolerance.
2.1.1. Chapter objectives

This chapter will look at the effect of low concentrations of hydrogen peroxide ($H_2O_2$), chlorhexidine (CHG), benzalkonium chloride (BKC) and glutaraldehyde (GTA) on *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442 and *Staphylococcus aureus* ATCC 6538.

*Escherichia coli* ATCC 8739. This ATCC strain was selected due to its recommended use in preparatory test control for antimicrobial handwashing formulations, antimicrobial preservatives and media testing (American Type Culture Collection 2016). *Escherichia coli* is a Gram-negative bacterium commonly transmitted to humans via the consumption of contaminated food and water (Kaper et al. 2004) and known to cause infections such as diarrhoea and urinary tract infections (Brzuszkiewicz et al. 2011). Outer membrane protein modification like the down regulation of porins and up regulation of efflux pumps such as AcrAB have been linked to increased antimicrobial resistance in this bacterium (Ma et al. 1993; Ma et al. 1994).

*Staphylococcus aureus* ATCC 6538: This ATCC strain is recommended for the testing of disinfectants, hand washing formulations, sanitisers and bactericides (American Type Culture Collection 2016).

*Staphylococcus aureus* is a Gram-positive, opportunistic pathogen responsible for both hospital and community acquired infections (Boucher et al. 2010; Latimer et al. 2012). Over expression of *qacA* to *qacH* pumps (Rouch et al. 1990) and NorA, NorB and NorC
Efflux pumps (Truong-Bolduc et al. 2006) have been linked antimicrobial resistance in this bacterium.

**Pseudomonas aeruginosa (ATCC 15442):** This is a quality control strain for testing antimicrobials hand washing formulations, disinfectants and bactericides (American Type Culture Collection 2016). *Pseudomonas aeruginosa* is an opportunistic, intrinsically resistant, Gram-negative bacteria that is responsible for a range of infections to the eyes and ears to serious complications in cystic fibrosis and bronchiectasis patients (Lambert 2002; Soothill 2013). Efflux pump upregulation such as MexAB, MexCD and MexEF and outer membrane protein modification have been linked to antimicrobial resistance in *P. aeruginosa* (Schweizer 1998; Poole 2001).

The strains will be gradually exposed to increasing concentrations of biocides using both the step wise and gradient plate methods. Increased tolerance to biocides will be tested by measuring minimum inhibitory concentration, and cross-resistance to antibiotics will be tested using both antibiotic disc diffusion test and measuring changes in minimum inhibitory concentrations.
2.2. Methods

2.2.1. Bacterial strains, storage and growth conditions

*Escherichia coli* (ATCC 8739), *P. aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (used in coagulase test only as a comparison organism), were stored on protect beads (Scientific Laboratory Supplies Limited Nottingham United Kingdom) at -80°C. Strains were grown for 24 hours in 10 ml of tryptone soya broth (TSB) (OXOID, Basingstoke, Hampshire, England) in a shaker incubator at 37°C at 3 x g. Prior to each experiment, frozen bacterial cells were grown on tryptone soya agar (TSA) at 37°C for 24 hours and single colonies were then sub-cultured on TSA at 37°C. Working cultures were kept at 4°C on TSA for up to three weeks, after which they were discarded, and new a culture was prepared.

2.2.2. Bacterial growth media and reagents

All growth media were obtained from OXOID, Basingstoke, Hampshire, England. Tryptone soya agar (TSA) and Mueller Hinton agar (MHA) were prepared by suspending agar powder 40 g and 38 g respectively in one litre of distilled water. Cetrimide agar was prepared by suspending 45.3 g agar powder and 10 ml glycerine in one litre distilled water, suspension was dissolved completely by microwaving. The agar suspensions were sterilised by autoclaving at 121°C, 15 pounds per square inch of pressure (psi) for 15 minutes, after which they were cooled to approximately 50°C before pouring into sterile Petri dishes and allowed to set at room temperature. Set plates were stored at 4°C.
Tryptone soya broth (TSB) and Mueller Hinton broth (MHB) were prepared by dissolving 30 g and 21 g respectively in one litre of distilled water. Each mixture was dispensed into glass universal bottles and sterilised by autoclaving at 121°C, 15 psi for 15 minutes after which cooled bottles were stored at room temperature.

Saline was prepared by dissolving 8.5 g sodium chloride (bacteriological grade) in one litre distilled water. The mixture was dispensed into glass universal bottles and autoclaved at 121°C, 15 psi for 15 minutes, after which they were cooled and stored at room temperature.

2.2.3. Biocides and concentration ranges

All biocide stock solutions were prepared daily. Hydrogen peroxide (H$_2$O$_2$) cat number 10386643 was obtained from Fisher Scientific Belgium in 500 ml volume, at a concentration of 30% v/v in water and stored at 4°C. Stock solutions were prepared in sterile distilled water and concentration range was 1-512 mg/l. Chlorhexidine gluconate contained in HiBi hand rub catalogue number NEXH3 (500 ml) at a concentration of 0.5% w/v (Molnlycke Health Care Ltd Manchester UK), working solutions were made directly from the liquid formulation by dilution in MH broth, concentration range used was 0.78 – 500 mg/l. Benzalkonium chloride cat number 12060-100G (100 g) was obtained from Sigma-Aldrich in semi-solid form at 95.0% and stored at 4°C. Stock solutions were prepared daily by dissolving required quantities in sterile distilled water, concentration range used was 1-512 mg/l. Glutaraldehyde cat number G5516-100ML (80 ml) was obtained from Sigma-Aldrich at a concentration of 25% in water and stored...
at -20 °C. Stock solutions were prepared daily by mixing required volumes in sterile
distilled water, concentration range used was 8 – 4096 mg/l.

2.2.4. Antibiotics and concentration ranges for minimum inhibitory
concentration test

All antibiotics were obtained from Sigma-Aldrich Gillingham United Kingdom and all
stock solutions were prepared daily. Oxacillin sodium salt (one gram) was stored at 4°C,
stock solutions were prepared by dissolving salt in sterile distilled water, concentration
range used was 0.0020 – 8 mg/l. Cefoxitin sodium salt (250 mg) was stored at 4°C, stock
solutions were prepared by dissolving salt in sterile distilled water, concentration range
used was 0.25 -32 mg/l. Cephalothin sodium salt (250 mg) and stored at 4°C, stock
solutions were prepared by dissolving salt in sterile distilled water concentration range
used was 0.25 - 32 mg/l. Ceftriaxone disodium salt hemi (heptahydrate) (250 mg) was
stored at 4 °C, working solution was prepared by dissolving salt in sterile distilled water,
concentration range used was 0.016 – 2 mg/l. Ampicillin one gram was obtained was
stored at 4°C , stock solution was prepared by dissolving salt in sterile distilled water,
concentration range used was 0.25 – 32 mg/l. Ciprofloxacin hydrochloride (400 mg) was
stored at 4°C, stock solution was prepared by dissolving salt in sterile distilled water,
concentration range used was 0.125 – 2 mg/l. Sulfathiazole sodium salt (100 g),
sulfadiazine sodium salt (25 g) and sulfamerazine sodium salt (250 g) were all stored at
room temperature. Each was dissolved in sterile distilled water to make stock solutions.
To prepare sulphatriad, working solutions of sulfathiazole, sulfadiazine and sulfamerazine were mixed in a percentage v/v/v ratio of 37:37:26.

All working solutions were prepared daily in sterile Mueller Hinton broth (MHB) as described in section 2.2.2.

2.2.5. Preparation of working solutions for minimum inhibitory concentration experiments.

Working solutions were prepared using a two-fold dilution series of each biocide and antibiotic, based on mg/l was prepared in Mueller-Hinton broth following the BS EN ISO 20776-6 guidelines/protocol (BSI 2006). Whereby 1 ml of stock solution was added to 9 ml MHB, 1 ml from this was added to different volumes of broth, to produce decreasing concentrations of the antibiotic. Working solutions were used immediately after dilution. Table 2.1, 2.2, 2.3 and 2.4 shows the dilution series for hydrogen peroxide glutaraldehyde, chlorhexidine and benzalkonium chloride.
5120 mg/l hydrogen peroxide was prepared in sterile distilled water, this was used to prepare a two-fold dilution series of working solutions in sterile Mueller Hinton broth (Table 2.1). Concentration range of 1-512 mg/l was used for MIC test.

Table 2.1. Dilution series for hydrogen peroxide.

<table>
<thead>
<tr>
<th>Stock (mg/l)</th>
<th>solution</th>
<th>Volume stock solution (ml)</th>
<th>Volume broth (ml)</th>
<th>Working solution (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5120</td>
<td>1</td>
<td>9</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td>1</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td>3</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td>7</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>1</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>3</td>
<td>16</td>
<td></td>
</tr>
<tr>
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<td>1</td>
<td>7</td>
<td>8</td>
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<td>4</td>
<td></td>
</tr>
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<td>1</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
40960 mg/l glutaraldehyde was prepared in sterile distilled water, this was used to prepare a two-fold dilution series of working solutions in sterile Mueller Hinton broth (Table 2.2). Concentration range of 8-4096 mg/l was used for MIC was test.

<table>
<thead>
<tr>
<th>Stock solution (mg/l)</th>
<th>Volume stock solution (ml)</th>
<th>Volume broth (ml)</th>
<th>Working solution (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40960</td>
<td>1</td>
<td>9</td>
<td>4096</td>
</tr>
<tr>
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<td>1</td>
<td>1</td>
<td>2048</td>
</tr>
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<td>4096</td>
<td>1</td>
<td>3</td>
<td>1024</td>
</tr>
<tr>
<td>4096</td>
<td>1</td>
<td>7</td>
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</tr>
<tr>
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<td>256</td>
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<tr>
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<td>1</td>
<td>3</td>
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<tr>
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<tr>
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<td>3</td>
<td>16</td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>
A dilution series of HIBI hand rub containing 500 mg/l chlorhexidine gluconate was prepared directly in sterile Mueller Hinton broth (Table 2.3), 0.78 - 50 mg/l was used for MIC test.

**Table 2.3 Dilution series of chlorhexidine gluconate**

<table>
<thead>
<tr>
<th>Stock solution (mg/l)</th>
<th>Volume stock solution (ml)</th>
<th>Volume broth (ml)</th>
<th>Working solution (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>1</td>
<td>9</td>
<td>50</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>1</td>
<td>25</td>
</tr>
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<td>3</td>
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<tr>
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<td>6.25</td>
<td>1</td>
<td>1</td>
<td>3.13</td>
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<td>6.25</td>
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<td>3</td>
<td>1.56</td>
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<tr>
<td>6.25</td>
<td>1</td>
<td>7</td>
<td>0.78</td>
</tr>
</tbody>
</table>
5120 mg/l benzalkonium chloride was prepared in sterile distilled water, this was used to prepare a two-fold dilution series of working solutions in sterile Mueller Hinton broth (Table 2.4). Concentration range of 8-512 mg/l was used for MIC was test.

Table 2.4 Dilution series of benzalkonium chloride

<table>
<thead>
<tr>
<th>Stock solution (mg/l)</th>
<th>Volume stock solution (ml)</th>
<th>Volume broth (ml)</th>
<th>Working solution (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5120</td>
<td>1</td>
<td>7</td>
<td>512</td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td>1</td>
<td>256</td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td>3</td>
<td>128</td>
</tr>
<tr>
<td>512</td>
<td>1</td>
<td>7</td>
<td>64</td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>
2.2.6. Preparation of inoculum Minimum inhibitory concentration and antibiotic susceptibility experiments.

**Colony suspension method**

To test each bacterial strain, for minimum inhibitory concentration and antibiotic susceptibility disc diffusion (section 2.27) four to five bacterial colonies were picked (to avoid selecting atypical variant) from a 37°C 24 h MHA plate and transferred into 10 ml sterile saline with a sterile wire loop. The suspension was mixed using a vortex mixer. The turbidity of the suspension was adjusted (with sterile saline) to the equivalence 0.5 McFarland standard, approximately 1x10⁸ CFU/ml (absorbance range between 0.08 – 0.13) by measuring with Spectramax plus spectrophotometer at a wavelength of 625 nm using semi-micro wavelength cuvette (Fisher brand). The resulting standardised suspension contained approximately 1x10⁸ CFU/ml. The standardised suspension was diluted in MHB in a 1:100 dilution, by transferring 0.1 ml to a sterile universal bottle containing 9.9 ml of Muller-Hinton broth to give an inoculum concentration of 1x 10⁶ CFU/ml in line with the European Committee for Antimicrobial Susceptibility Testing (EUCAST 2015) and of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), 2000).

2.2.7. Establishing minimum inhibitory concentration of biocides

The minimum inhibitory concentrations (MICs) of biocide and formulated products containing biocides were determined for each strain before exposure to low
concentrations of biocides, to establish as baseline to compare any potential isolate too and to identify suitable concentrations for use in the priming experiments (section 2.2.9). These were carried out using broth suspension method of testing in accordance with EUCAST guidelines. Briefly, 50 µl of the standardised inoculum was dispensed into each well of a 96-well plate already containing 50 µl of appropriate concentration of biocides or formulated products to give a final inoculum concentration of $5 \times 10^5$ colony forming units per ml (CFU/ml). Positive and negative controls consisting of 100 µl of the standardised inoculum and 100 µl of sterile broth respectively were dispensed into two wells of the 96-well plate during each experimental run. The inoculated 96-well plate was incubated at 37°C for 24 hours. The lowest concentration of biocide that prevented bacterial growth after 24 hours of incubation was used to establish the MIC. Bacteria growth was determined in two ways: by visual observation of growth on the 96-well plate and by measuring absorbance at 625 nm in Spectramax plus plate reader. The experiments were carried out as biological and technical triplicates (n=3).

Viable counts were performed on the test suspension to confirm the presence of approximately $5 \times 10^5$ CFU/ml and strain purity, this was conducted by removing 10 µl from the positive control well immediately after inoculation and diluting in 10 ml of Mueller-Hinton broth. Aliquots (100 µl) of the diluted positive control was spread onto the surface of Mueller-Hinton agar and incubated at 37°C for 24 hours before enumeration.
2.2.8. Antibiotic susceptibility testing using disc diffusion test

The antibiotic susceptibility of each bacteria strain was determined before treatment with biocides using the disc diffusion method of antimicrobial susceptibility testing. This was done in accordance to the guidelines of European committee on antimicrobial susceptibility testing (EUCAST). Briefly, entire surfaces of MHA plates were inoculated confluently with sterile cotton swab dipped in the standardised bacterial suspension previously described in section 2.2.6, excess fluid was removed by turning the swab against the inside of the universal bottle, before swabbing in three directions onto the surface of the agar. Antibiotic discs purchased from, Mast Group Ltd, Merseyside UK were applied to surfaces of the inoculated plates within 15 minutes of inoculation. After 24 hours of incubation at 37°C, zones of inhibition were measured against a dark background, illuminated with reflected light, using a digital Vernier calliper. Tests were repeated three times (n=3).

2.2.9. Priming of bacteria strains with biocides

**Stepwise method**

The stepwise training method was used to attempt to select for bacteria with increased tolerance to biocides. For this test, the starting concentration was the concentration just below the minimum inhibitory concentration (MIC) for each biocide against each strain. The inoculum was prepared as described in section 2.2.6, 160 µl of Mueller-Hinton broth
was pipetted into a well of a 96-well plate (with lid), along with 20 µl of working solution described in section 2.2.5 and 20 µl standardised inoculum described in section 2.2.6. The well plate was covered and incubated at 37°C for 24 hours. When growth was observed, 20 µl from the well with growth was aseptically transferred to the next well, which already contained 20 µl of working solution (biocide) and 160 µl of Mueller-Hinton broth. The concentration of each well increased to 1.5 times the concentration of the previous well after each transfer. Transfer was carried out every 24/48 hours until no growth was observed, after which the well with the last observed growth was streaked onto TSA plate (prime plate) and incubated at 37°C for 24 hours. After 24 hours a colony was selected from the prime plate, the colony was streaked onto in tryptone soya agar plate and incubated at 37 °C for 24 hours. Colonies from the plate were tested for increased tolerance to biocides as described in section 2.2.7 and cross-resistance with antibiotics as described in section 2.2.8.

**Gradient plate method**

A second method using Petri-dishes containing a concentration gradient of the individual biocides was also used to attempt to select for tolerance. A two fold dilution series of biocides were prepared in sterile distilled water following the EUCAST guidelines (European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) 2000). One ml of required biocide was added to 19 ml sterile molten nutrient agar to give specific final concentrations (from a concentration just below the MIC of the biocide against tested bacteria strain). The molten agar and biocide mixture were poured into sterile
Petri dishes and set at an angle, after which plates were placed on a flat surface and 20 ml sterile molten agar were poured over the first layer and allowed to set. Plates were left at 4°C for 24 h to allow diffusion of biocide, diagram illustrating how this is made is shown in Figure 2.1. Bacterial cultures (24h) grown in MHB at 37°C in a shaker incubator were streaked along the concentration gradient starting at the point of the gradient plate containing the lowest concentration of biocide. Streaked plates were incubated at 37 °C for 24 hours. Bacteria colonies that grew the furthest along the gradient towards the high concentration were used to inoculate a second gradient plate containing a higher concentration of biocide, this process of sub culturing onto new gradient plates with increasing biocide present, was repeated until no further increases in the length of growth along the gradient was observed. The furthest growing bacteria from the last plate were streaked onto a biocide-free TSA plate (primed plate) and incubated at 37°C for 24 hours. A colony from the primed plate was spread onto a TSA plate and incubated at 37 °C for 24 hours. Colonies from the plate was tested for change in biocide and antibiotic profiles as described in stepwise method (section 2.2.9).
Figure 2.1. Preparation of biocide gradient plates

Illustration of the preparation of biocide gradient plate; (a) sterile petri dish was set at an angle, (b) molten agar containing required concentration of biocide was poured in, covered and left to set, (c) Set plate was placed on a flat surface and 20 ml of molten agar was poured over the first layer and (d) the plate was covered and left to set at room temperature. Prepared gradient plates were kept in 4°C for 24 hours to allow diffusion of biocide.
2.2.10. Testing for increased tolerance to biocides

Colonies from previously streaked primed plate prepared in section 2.2.9 from either stepwise or gradient plate method were used to prepare an inoculum as described in section 2.2.6 and were then tested to check for any changes in MIC to the biocide. This was carried out using the same method as used for establishing the MICs of the standard strains (as described in section 2.2.7). Tests on each plate were conducted as biological and technical triplicates (n=3).

2.2.11. Stability of adaptive resistance and tolerance

To confirm the stability of the isolates produced during the training (sections 2.2.9), one colony from the streaked plate in section 2.2.9 was sub-cultured 15 times in 10 ml of biocide-free TSB. Antibiotic susceptibility between parent and primed strains was tested and compared after subculture; one, two, 10 and 15, following EUCAST disc diffusion test previously described sections 2.2.8. The 15th subculture was streaked onto tryptone soya agar plate and incubated at 37 °C for 24 hours before testing for changes in minimum inhibitory concentration and antibiotic susceptibility as described in sections 2.2.7, 2.2.8 and 2.2.12. One colony from the plate was stored on protect beads at 80°C. The plate was stored in 4°C for up to three weeks to be used for minimum inhibitory concentration and biochemical tests (2.2.12 and 2.2.13) after which it was discarded, and fresh plate was made from the isolate that was stored in -80°C. All isolates exposed to biocides were tested for increased tolerance to biocide and cross-resistance to antibiotics, however only isolates showing changes were kept for further investigation.
2.2.12. Biochemical tests of stable isolates

To confirm the identity of the isolates was the same as the parent strains, the following tests were performed.

**Gram staining**

Individual colonies from 24-hour tryptone agar plates were Gram stained to compare both parent and primed strains.

**Analytical profile index 20E (API20E) test**

Single colony of the *E. coli* isolate that had been trained in the presence of H$_2$O$_2$ (EcH2O2) from 24-hours tryptone soya agar plate was added to 10 ml of sterile distilled water and vortexed to make a suspension. The bacterial suspension was used to fill the wells of an analytical profile index 20E (API20E) test (Biomerieux) strip with a sterile pasture pipette. Sterile liquid paraffin was used to top the wells marked ADH, LDC, ODCH25 and URE, drops of sterile distilled water was added to the strip tray, the filled API strip was placed on to and the tray was covered with its lid. The tray was incubated at 37°C for 24 hours.

After 24 hours, reagents were added to the wells labelled TDA, IND, VP following manufacturer’s instructions. The scores of positive wells were added in triplet and three test reactions were added together to give a seven-digit number which was used to identify the organism online using apiweb.
Growth of organisms in cetrimide agar

Colonies of parent and biocide primed *P. aeruginosa* from a 24h TSA plate grown at 37°C were streaked onto cetrimide agar plates and incubated at 37 °C for 48 hours. Corresponding *E. coli* plates were prepared and incubated alongside. Growth was observed after 48 hours.

**Oxidase test**

An oxidase strip (Fisher scientific Loughborough) was used to touch single colony of parent and primed strains *P. aeruginosa* on a 24 h tryptone agar plate grown at 37°C. The strips were observed for colour change. The *E. coli* parent and primed stains were performed alongside for comparison.

**Catalase test**

A sterile wire loop was used to transfer a single colony of *S. aureus* parent and primed strains from a 24h TSA plate grown at 37°C onto a clean glass slide. One drop of 3 % H₂O₂ was placed onto the slide with a sterile pasture pipette and mixed, slides were observed for bubbles after five to ten seconds.

**Coagulase test**

A drop of physiological saline was placed on 2 clean glass slides. A sterile straight wire was used to pick a colony from 24h plates of either parent or primed strain *S. aureus*. After emulsifying, a drop of rabbit plasma (Fisher Scientific Loughborough) was added
to each plate, mixed and gently rocked. After 10 seconds the slides were observed for clumping. A sample slide with *S. epidermidis* was prepared alongside as negative control.

2.2.13. Genomic DNA extraction

A single colony from a TSA plate (grown at 37 °C for 24 hours) was isolated and inoculated in 5 ml sterile TSB and incubated at 37°C overnight with shaking at 3 x g. Cultures were centrifuged at 300 x g for 5 minutes, supernatant was discarded and pellets was resuspended in 200 µl phosphate buffered saline (PBS), 25 µl (15 mAU) protein kinase K and 200 µl binding buffer (AL) and incubated at 70°C for 10 minutes. Genomic DNA was extracted from both parent and primed strains of bacteria using the Qiagen DNeasy® Blood & Tissue kit following manufacturer’s instructions. Extracted DNA was stored at -20°C.

2.2.14. Measurement of DNA concentration

One microliter of DNA was measured and quantified at absorbance of 260 nm using nanodrop (Nanodrop Lite Spectrophotometer, Thermo Fisher Scientific, Loughborough, UK), after calibration with 1 µl nuclease free water.

2.2.15. Polymerase chain reaction (PCR)

16S rRNA genes sequences were amplified from parent *E. coli* ATCC 8739, EcH2O2, *S. aureus* ATCC 6538 and SaCHG strains using universal primers; AGAGTTTGATCCTGGCTCAG (forward) and ACGGCTACCTTGTTACGACTT (reverse). Universal primers were supplied by Universal DNA Technologies (IDT), Belgium.
Polymerase chain reaction was carried out in a Labnet multigene™ optimax thermal cycler, following the typical reaction profile shown below, unless otherwise stated.

Table 2.5. PCR reaction volumes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volumes (µl)</th>
</tr>
</thead>
<tbody>
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<td>Forward primer (100 pmol/µl)</td>
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</tr>
<tr>
<td>Reverse primer (100 pmol/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Genomic DNA (~0.1 µg/µl)</td>
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</tr>
<tr>
<td>Q5® Hot Start 2X Master Mix (New England Biolabsinc®).</td>
<td>25</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>22</td>
</tr>
</tbody>
</table>

**Cycling parameters**

- **Initial denaturation**: 95°C – 5 minutes
- **Denaturation**: 98°C - 30 seconds
- **Annealing**: 50 °C -30 seconds
- **Extension**: 72 °C -60 seconds
- **Final extension**: 72°C -7 minutes.
2.2.16. Agarose gel electrophoresis

Agarose gel electrophoresis was used for visualizing DNA fragments. Agarose 1 % (w/v) (Thermo Fisher) was added to 1X TBE buffer containing 0.5X SYBR™ Safe DNA Gel Stain (Thermo fisher) and melted in a microwave oven. It was left to cool down, after which it was set in a casting tray with comb in place. Prior to loading, 5 µl of the PCR product were mixed with 6X loading dye (Thermo Fisher). A 1 kb ladder GeneRuler (Thermo Fisher) was used to calibrate the DNA fragments and gel electrophoresis was carried out in 1X TBE buffer at 5V/cm for 1-hour in. GelDoc system (Biorad) was used to visualize the DNA fragments.

2.2.17. 16S rRNA sequencing

50 µl each of amplified DNA and 30 µl of 16S rRNA primers (both forward and reverse) at 10 pmole/µl each were sent to Macrogen Europe for sequencing. Results were analysed using NCBI BLAST.
2.3. Results

2.3.1. Establishing minimum inhibitory concentration of biocides

The minimum inhibitory concentrations of biocides against wild type strains (EcATCC, SaATCC and PaATCC) were established before priming with low concentrations of biocides (H\textsubscript{2}O\textsubscript{2}, CHG, GTA and BKC). The results are summarised in Table 2.6. The MIC for hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was in the range of (16-32 mg/l) for \textit{E. coli} ATCC 8739 (EcATCC) and \textit{P. aeruginosa} ATCC 15442 (PaATCC) but was lower range of (4 -8 mg/l) for \textit{S. aureus} ATCC 6538 (SaATCC). The MIC for glutaraldehyde (GTA) for each strain were highest for EcATCC and PaATCC (1024 mg/l), with lower levels for SaATCC at 512 mg/l. For benzalkonium chloride (BKC), the highest MIC was recorded with PaATCC (64 mg/l), whilst EcATCC and SaATCC had MICs of 16 mg/l and 4 mg/l respectively. For chlorhexidine (CHG) the MIC for EcATCC was in a range of 0.19 to 0.39 mg/l, with SaATCC having MIC in the range of 0.39 to 0.78 mg/l and PaATCC an MIC range of 1.56 mg/l.

2.3.2. Testing for increase tolerance to biocides

After exposure to low concentrations of biocides by either stepwise or gradient plate method the MIC of the biocides were measured against treated strains to see if there was an increase in tolerance. The stepwise method did not yield tolerant or resistant strains in this study (results not shown).

The results for selected gradient plate isolates are shown in Table 2.6 (please note that although all isolates were tested, but only those demonstrating tolerance or resistance
are shown in the table), \(H_2O_2\) primed \textit{E. coli} (EcH2O2) showed a two-fold increase in MIC (from 32 mg/l to 64 mg/l) to the biocide, this was however unstable as it reverted after six weeks of storage at 80°C. Benzalkonium chloride primed \textit{P. aeruginosa} (PaBKC) also showed a stable two-fold increased MIC to the biocide, from 64 mg/l to 128 mg/l, indicating increased tolerance.

**Table 2.6. MIC of biocides against primed bacteria strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>(H_2O_2) (mg/l)</th>
<th>(GTA) (range)</th>
<th>(BKC) (range)</th>
<th>(CHG) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcATCC</td>
<td>32 (16 - 32)</td>
<td>1024 (512-1024)</td>
<td>16 (16-32)</td>
<td>0.39 (0.19 - 0.39)</td>
</tr>
<tr>
<td>EcH2O2</td>
<td>64*</td>
<td>1024 (512-1024)</td>
<td>16*</td>
<td>0.39 (0.19 - 0.39)</td>
</tr>
<tr>
<td>SaATCC</td>
<td>4 (4 - 8)</td>
<td>512 *</td>
<td>4 (2-4)</td>
<td>0.78 (0.39 - 0.78)</td>
</tr>
<tr>
<td>SaCHG</td>
<td>4 (4 - 8)</td>
<td>512*</td>
<td>4 (4-8)</td>
<td>0.78 (0.39 - 0.78)</td>
</tr>
<tr>
<td>PaATCC</td>
<td>32*</td>
<td>1024*</td>
<td>64*</td>
<td>1.56*</td>
</tr>
<tr>
<td>PaGTA</td>
<td>32*</td>
<td>1024*</td>
<td>64*</td>
<td>1.56*</td>
</tr>
<tr>
<td>PaBKC</td>
<td>32*</td>
<td>1024*</td>
<td>128*</td>
<td>1.56*</td>
</tr>
</tbody>
</table>

*, where no range is present, result was always that reported, n=3.
2.3.3. Testing for cross-resistance to antibiotics using disc diffusion test

After exposure to low concentrations of biocides, the EUCAST disc diffusion method was used to test for change in antibiotic resistant profile of primed strains (EcH2O2, SaCHG, PaGTA and PaBK) compared to that of wild type (EcATCC, SaATCC and PaATCC). (please note that although other isolates were tested, only the results for the seven primed strains that demonstrated cross-resistance to antibiotics are shown in Table 2.7. A sample of the results of antibiotic susceptibility tests are shown in Figure 2.2 and the full results are summarised in Table 2.8. There was no zone of inhibition around cephalothin, sulphatriad and ampicillin discs in EcH2O2 (a) indicating a cross-resistance to the antibiotics after exposure to low concentrations of H₂O₂, (b) compared to 9.78 mm, 28.03 mm and 14.74 mm respectively (c). Glutaraldehyde primed P. aeruginosa (PaGTA) showed no zone of inhibition around sulphatriad disc (d) compared to PaATCC 15.11 mm zone (c). There was no zone of inhibition around oxacillin disc in chlorhexidine primed S. aureus (SaCHG) (f) compared to SaATCC 25.74 mm zone (e).
Figure 2.2 Antibiotic resistant profiles of bacterial strains following biocide priming

EUCAST disc diffusion test was used to test the change in antibiotic resistant profile of wild-type and biocide primed strains. (a) For EcATCC, there was zones of inhibition around ampicillin, sulphatriad and cephalothin discs (9.78 mm, 20.03 mm and 14.74 mm) respectively, compared to, (b) EcH2O2, there was no zone of inhibition around ampicillin, sulphatriad and cephalothin discs indicating cross-resistance to the antibiotics (c) For PaATCC, there was 15.11 mm a zone of inhibition around sulphatriad disc, compared to (d) PaGTA showing no zone of inhibition around sulphatriad disc, indicating cross-resistance to sulphatriad. (e) For SaATCC, there was 25.74 mm zone of inhibition around oxacillin disc, compared to (f) SaCHG, showing no zone of inhibition around oxacillin disc indicating cross-resistance to oxacillin. (n=3).
Table 2.7 Antibiotic susceptibility profiles of pre and post adapted strains

<table>
<thead>
<tr>
<th>Antibiotics (µg)</th>
<th>EcaTCC</th>
<th>Ech202</th>
<th>SaATCC</th>
<th>SaCHG</th>
<th>PaATCC</th>
<th>PaSTA</th>
<th>PaSBCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin (10)</td>
<td>14.94(1.20)</td>
<td>*NZ</td>
<td>CZ</td>
<td>CZ</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Cephalothin (5)</td>
<td>9.78(0.92)</td>
<td>*NZ</td>
<td>CZ</td>
<td>CZ</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Colestin (25)</td>
<td>13.99(0.20)</td>
<td>13.77(0.58)</td>
<td>NZ</td>
<td>NZ</td>
<td>15.15(0.89)</td>
<td>14.36(0.84)</td>
<td>13.86(0.36)</td>
</tr>
<tr>
<td>Gentamicin (10)</td>
<td>18.15(0.66)</td>
<td>17.53(0.73)</td>
<td>21.86(0.52)</td>
<td>21.59(0.72)</td>
<td>17.10(0.17)</td>
<td>17.72(0.62)</td>
<td>13.23(0.20)</td>
</tr>
<tr>
<td>Streptomycin (10)</td>
<td>15.34(0.22)</td>
<td>13.98(1.72)</td>
<td>16.99(0.29)</td>
<td>15.37(2.47)</td>
<td>10.86(0.46)</td>
<td>11.81(0.15)</td>
<td>7.2(3.60)</td>
</tr>
<tr>
<td>Sulphadiazine (200)</td>
<td>28.93(1.01)</td>
<td>*NZ</td>
<td>21.12(2.33)</td>
<td>17.83(1.16)</td>
<td>15.11</td>
<td>*NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Tetracycline (25)</td>
<td>21.27(1.07)</td>
<td>23.26(1.54)</td>
<td>27.54(0)</td>
<td>26.22(2.62)</td>
<td>6.66(0.46)</td>
<td>7.91(0.63)</td>
<td>10(0.34)</td>
</tr>
<tr>
<td>CoTrimoxazole (200)</td>
<td>29.07(1.04)</td>
<td>18.22(1.55)</td>
<td>CZ</td>
<td>CZ</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>C (25)</td>
<td>18.8(1.72)</td>
<td>*14.41(2.00)</td>
<td>20.76(0.97)</td>
<td>17.8(0.82)</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Erythromycin (5)</td>
<td>NZ</td>
<td>NZ</td>
<td>19.86(1.77)</td>
<td>17.58(0.15)</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Fusidic acid (10)</td>
<td>NZ</td>
<td>NZ</td>
<td>22.04(1.65)</td>
<td>23.05(1.5)</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Oxacillin (5)</td>
<td>NZ</td>
<td>NZ</td>
<td>25.74(2.98)</td>
<td>*NZ</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Norfloxacin (5)</td>
<td>NZ</td>
<td>NZ</td>
<td>20.45(1.05)</td>
<td>20.99(0.70)</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Penicillin G (1 unit)</td>
<td>NZ</td>
<td>NZ</td>
<td>27.57(2.89)</td>
<td>26.16(1.50)</td>
<td>NZ</td>
<td>NZ</td>
<td>NZ</td>
</tr>
<tr>
<td>Streptomycin (10)</td>
<td>16.95(0.18)</td>
<td>13.65(1.47)</td>
<td>17.06(0.32)</td>
<td>14.37(0.61)</td>
<td>10.85(0.39)</td>
<td>9.93(0.69)</td>
<td>10.87(0.34)</td>
</tr>
<tr>
<td>Tetracycline (25)</td>
<td>23.45(1.88)</td>
<td>22.84(1.01)</td>
<td>29.40(2.40)</td>
<td>27.16(0.59)</td>
<td>8.43(0.44)</td>
<td>8.20(0.39)</td>
<td>7.62(0.27)</td>
</tr>
</tbody>
</table>

NZ = no zone around disc, CZ = complete clear zone around disc, *= Cross-resistance observed.
2.3.4. Stability of adaptive resistance and tolerance

To confirm the stability of the observed increase in tolerance to biocides and cross-resistance to antibiotics, EcH2O2, PaBKC, PaGTA and SaCHG were passaged 15 times in biocide and antibiotic free tryptone soya broth. Minimum inhibitory concentration and disc diffusion tests were repeated to see if the changes observed remained.

In EcH2O2 the two-fold increase in tolerance to H₂O₂ was stable after 15 sub-cultures in biocide-free broth (but was lost after storage at -80°C for six weeks), cross-resistance in this isolate to ampicillin, sulphatriad and cephalothin remained stable. In PaBKC, the two-fold increase tolerance to benzalkonium chloride was stable, but no changes in antibiotic susceptibility were seen. The cross-resistance to sulphatriad and oxacillin seen in PaGTA and SaCHG respectively were also stable.

2.3.5. Minimum inhibitory concentration of antibiotics

After reduced susceptibility to antibiotics using disc diffusion test and stability of resistance were established, potential changes in susceptibility to other antibiotics were investigated. The strains and antibiotics selected for testing were based on the results obtained from antibiotic disc diffusion tests. In E. coli due to the increased tolerance of EcH2O2 to cephalothin (a first-generation cephalosporin) both cefoxitin and ceftriaxone, second and third generations cephalosporins were included in further MIC testing to see if the cross-resistance extended to newer antibiotics. In P. aeruginosa because of reduced susceptibility of PAGTA to sulphatriad MIC to both sulphatriad and ciprofloxacin
were tested and MIC of oxacillin against SACHG was also tested to confirm cross-resistance observed.

Results of MIC changes in primed strains compared to parent strains are summarised in Table 2.8. There was an eight-fold increase in the MIC of EcH2O2 to cephalothin, from 4 mg/l to 32 mg/l, a four-fold increase in MIC to cefoxitin from 4 mg/l to 16 mg/l and a two-fold increase MIC to ceftriaxone, from 0.0625 mg/l to 0.125 mg/l. An increase in MIC from 2 mg/l to 4 mg/l to ampicillin was also observed in EcH2O2. The MIC breakpoint (EUCAST 2020) for Enterobacteria reports resistance at >2 mg/l with ceftriaxone indicating no change in clinical susceptibility, EUCAST data for cephalothin and cefoxitin are not available on the EUCAST site.

A four-fold increased MIC to oxacillin was observed in SaCHG from 0.5 mg/l to 2 mg/l (breakpoint data not available), confirming the cross-resistance observed from the disc diffusion test.

In PaGTA, an eight-fold increase in MIC to ciprofloxacin was observed, 1 mg/l compared to PaATCC 0.125 mg/l and a four-fold increased MIC to sulphatriad was also recorded, confirming the initial cross-resistance results observed in disc diffusion test. The MIC breakpoint (EUCAST 2020) for Pseudomonas spp. and ciprofloxacin reports resistance at >0.5 mg/l indicating clinically significant resistance in PaGTA, breakpoint data for sulphatriad is not available on the EUCAST site.
### Table 2.8 MICs of selected antibiotics against parent and primed strains bacteria

<table>
<thead>
<tr>
<th>Stains</th>
<th>MIC (mg/l), mode n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cephalothin</td>
</tr>
<tr>
<td>EcATCC</td>
<td>4</td>
</tr>
<tr>
<td>EcH2O2</td>
<td>32</td>
</tr>
<tr>
<td>SaATCC</td>
<td>-</td>
</tr>
<tr>
<td>SaCHG</td>
<td>-</td>
</tr>
<tr>
<td>PaATCC</td>
<td>-</td>
</tr>
<tr>
<td>PaGTA</td>
<td>-</td>
</tr>
</tbody>
</table>

-, Not tested
2.3.6. Biochemical tests of stable isolates

To confirm the identities of primed isolates, several biochemical tests plus a Gram stain was carried out. Analytical profile index 20E (API20E) test for EcH2O2 identified it as 94.7 % *Escherichia coli*. Results of other biochemical tests are were also consistent with their presumed identity. Further tests including Gram staining, cetrimide agar growth, oxidase, catalase and coagulase tests gave the expected results for EcH2O2, SaCHG and PaGTA. The results of biochemical tests and Gram-stain for stable primed strains are summarised in Table 2.9.

**Table 2.9. Biochemical tests of stable isolates**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gram stain</th>
<th>Cetrimide growth</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Coagulase</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcATCC</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>EcH2O2</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>PaATCC</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>PaGTA</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>PaBK</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>SaATCC</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SaCHG</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, Positive. -, Negative. NT, Not tested.
2.3.7. PCR and 16S rRNA sequencing

In order to confirm the identities of the antibiotic resistant isolates generated by gradient plate method (section 2.29), 16S rRNA primers AGAGTTTGATCCTGGCTCAG and ACGGCTACCTGTTACGACTT were used to run PCR (section 2.2.16) 1 % agarose gel electrophoresis was used to separate PCR products. Amplified PCR products along with 16Sr RNA primers were sent to Macrogen for sequencing (section 2.2.17). Figure 2.3 (a) primers used in the reaction bound to; lane 2 and 3, EcATCC and EcH2O2 16sR RNA gene, Lane 4 and 5 primers did not bind to PaATCC, PaGTA 16Sr RNA gene, lane 6 and 7 SaATCC and SaCHG 16Sr RNA gene. Expected amplicon size was 1505 bp for E. coli strains, 1494 bb for S. aureus strains and 1500 bp for P. aeruginosa strains. Figure 2.3 (b), BLAST results of 16S rRNA sequence showed over 98 % similarity between EcH2O2, E. coli ATCC 8739 and other strains of E. coli and 99 % similarity between SaCHG, S. aureus ATCC 6538 and other S. aureus strains. The parameters and the universal 16S rRNA primers used for E. coli and S. aureus strains failed to amplify 16S rRNA gene in PaATCC and PAGTA due to impactable nucleotide sequence along the primer binding site. Adjusting the PCR cycling parameters did not change the outcome.
Figure 2.3 Gel electrophoresis and sequence analysis of 16S rRNA gene

Gene specific primers were used for each PCR reaction, products were separated by gel electrophoresis on 1 % agarose gel. Amplified PCR product was sequenced and analysed with NCBI BLAST. (a), Primers used in the reaction corresponds with; Lane 1 50 bp ladder, lane 2 and 3 (EcATCC and EcH2O2) lane 4 and 5 primers did not bind to (PaATCC, lane 5 PaGTA), and lane 6 and 7 (SaATCC and SaCHG) (expected amplicon size 1505 bp for E. coli, 1500 bp for S. aureus and 1494 bp), (b) SaATCC and SaCHG showed over 99 % sequence similarity and EcATCC and EcH2O2 showed over 98 % similarity.
2.4. Discussion

The goal of this chapter was to test the priming effects of sub-inhibitory concentrations of four biocides; hydrogen peroxide (H$_2$O$_2$), glutaraldehyde (GTA), benzalkonium chloride (BKC) and chlorhexidine (GTA) on selected strains of bacteria (*E. coli*, *P. aeruginosa* and *S. aureus*). Bacterial strains were exposed to low concentrations of biocides in a gradient dependent way. Of all the strains tested only *P. aeruginosa* showed stable 2-fold increased tolerance to BKC, an initial stable 2-fold increased tolerance of *E. coli* to H$_2$O$_2$ was observed, however this was lost after storage at -80°C for six weeks. There was stable cross-resistance to β-Lactams in H$_2$O$_2$ primed *E. coli*, oxacillin in CHG primed *S. aureus*, sulphatriad and ciprofloxacin in GTA primed *P. aeruginosa*.

Low concentrations of H$_2$O$_2$ have previously been shown to promote tolerance in bacteria (Imlay and Linn 1987; Bogomolnaya *et al.* 2013) as was initially seen in this study. In the present study the increased tolerance to H$_2$O$_2$ to *E. coli* from 32 mg/l to 64 mg/l was short lived (*Table 2.6*), however stable cross-resistance to cephalothin, cefoxitin, ceftriaxone and ampicillin antibiotics was observed *Figure 2.2 (b)*, *Table 2.7 and 2.8*. Previous studies showed H$_2$O$_2$ as an activator of both soxRS and the oxyR regulons (Aslund *et al.* 1999, Manchado *et al.* 2000), both of which control the expression of over 100 oxidative stress response genes (Demple, Bruce 1991; Blanchard *et al.* 2007; Blanchard *et al.* 2012) such as the multi-drug efflux genes the acrAB (Dukan *et al.* 1996; Storz and Imlayt 1999). Exposing *E. coli* to sub-inhibitory concentrations of H$_2$O$_2$ may trigger the activation of protective enzymes such as catalases and peroxidases.
to decompose hydrogen peroxide and protect the cell from oxidative damage by reactive oxygen species *E. coli* (Finn and Condon 1975; Richter and Loewen 1981; Pomposiello and Demple 2001, Chiang and Schellhorn 2012). Expression of both catalases and peroxidases such as the cytochrome c peroxidase in (Khademian and Imlay 2017), are controlled by the *soxRS* operon which can be activated by H$_2$O$_2$ and which controls the expression of other antibiotic resistances genes such as those that encode for efflux pumps and porins (Demple 1991; Blanchard *et al.* 2007; Blanchard *et al.* 2012). This may explain the cross-resistance to antibiotics observed here after H$_2$O$_2$ priming. These results will be further tested to see if indeed either *soxRS* or *oxyR* regulons were activated. This will be carried out by comparing the expression levels of genes involved in both parent and primed strains.

While previous studies show that both high and low concentrations of CHG can cause increase resistance in both Gram-negative and Gram-positive bacteria (Wand *et al.* 2016; Hardy *et al.* 2017). As demonstrated in the 2016 work carried out by Wand *et al.*, where they observed resistance to CHG and cross-resistance with colistin in *K. pneumoniae* following exposure of the bacteria to low concentrations of CHG (Wand *et al.* 2016) and the work of Hardy *et al.*, where prolonged exposure of *S. aureus* isolates to CHG led to reduced susceptibility to the biocide (Hardy *et al.* 2017).

In the present study, exposure of both Gram-positive and Gram-negative bacteria to low concentrations of CHG did not cause increased tolerance to the biocide in all the strains studied, there was however a cross-resistance to oxacillin observed in *S. aureus* strain exposed to the biocide (*Figure 2.2 and Table 2.7*). Exposure of *S. aureus* to CHG did not
cause increased tolerance to the biocide in the present study, contrasting the findings of Hardy et al. where prolonged exposure of S. aureus isolates to CHG led to reduced susceptibility to the biocide (Hardy et al. 2017).

The activation of multidrug efflux systems such as the RND-type MexCD-OprJ was previously linked to the use of sub-inhibitory concentrations of membrane damaging biocides like BKC and CHG (Morita et al. 2003; Fraud et al. 2008). A previous adaptation of P. aeruginosa to sub-inhibitory concentrations of BKC caused the activation of efflux mechanism in the bacteria (Loughlin et al. 2002; Mc Cay et al. 2010).

The exposure of P. aeruginosa to low concentrations of BKC in the present study, caused a stable two-fold increased tolerance to the biocide. The data presented here agrees in parts with that of Mc Cay et al. where they observed a 12-fold increased tolerance to the biocide and a cross-resistance with ciprofloxacin when P. aeruginosa was grown in continuous culture (Mc Cay et al. 2010). Our findings are however slightly different from that of Loughlin et al. where cross-resistance was observed with other quaternary ammonium compounds after serial batch culture (Loughlin et al. 2002). The varied outcome in these studies shows how different growth conditions may influence adaptation and selection of bacteria to antimicrobials.

There are several reports of P. aeruginosa resistance to GTA (Kirschke et al. 2003; Simoes et al. 2006; Svetlikova et al. 2009; Simões et al. 2011; Tschudin-Sutter et al. 2011; Kampf et al. 2013), which was not observed in this study. However, cross-resistance with ciprofloxacin and sulphatriad was observed here Figure 2.2 (d), Table 2.7 and 2.8 after
priming with GTA. The MIC breakpoint (EUCAST 2020) for *Pseudomonas spp.* and ciprofloxacin reports resistance at >0.5 mg/l, indicating clinically significant resistance in PaGTA. Another study using *P. aeruginosa* and *P. fluorescens* biofilms showed that their exposure to GTA could induce phosphonate degradation, lipid biosynthesis and the upregulation of two multidrug efflux genes (PFLU2929 and PFLU3876) (Vikram et al. 2015). The cross-resistance observed with sulphatriad and ciprofloxacin antibiotics in this study potentially suggest that efflux is involved as seen previously (Poole 2002; Maseda et al. 2009; Sanchez et al. 2005; Ferreira et al. 2011) where exposure to antimicrobials resulted in the upregulation of efflux mechanisms.

2.4.1. The effect of biocide priming

While cross-resistance to antibiotics and corresponding tolerance to biocides after biocides exposure has been reported (Slayden et al. 2000; Chuanchuen et al. 2001; Braoudaki and Hilton 2004a; Tkachenko et al. 2007; Soumet et al. 2012; Mavri and Možina 2012; Wand et al. 2016). The present study shows cross-resistance to antibiotics after exposure to biocide with no observable corresponding biocidal tolerance. The data obtained in this study indicates that the low concentrations of biocides triggers the activation of protective response mechanisms in bacteria. This may explain the initial stable increase in MIC to H$_2$O$_2$ in EcH2O2, which was lost after storage for six weeks. The loss of increased tolerance to H$_2$O$_2$ did not affect the stable cross-resistance to β-lactam antibiotics, suggesting that the biocide was acting as a priming agent for resistance.
Priming is clinically exploited in cancer chemotherapy where cells are exposed to low concentrations of toxic agents to prepare them against future lethal doses of the same agent (Kimball et al. 1976; Collis et al. 1980). Exposure to continuous environmental stress can promote memory (priming) in most biological systems (Hilker et al. 2016; Rodríguez-Rojas et al. 2019). For example, the continuous stress conditions bacteria are exposed to from host immune system during an infection may affect their response during future encounters (Wittmann et al. 2012). The introduction of low-level oxidants such as methyl viologen dichloride hydrate, into the guts of Drosophila melanogaster was previously demonstrated to influence resident microbiota and affect the lifespan of the flies (Obata and Gould 2018).

As observed in this present study, exposure to low concentration of H₂O₂ (60 µM) was previously shown to increase the survival of E. coli in higher concentration (30 mM) of the biocide (Imlay and Linn 1986). Rodríguez-Rojas et al in their 2019 study showed that not only did exposure to low concentrations prepare bacteria for surviving subsequent lethal doses, the effect was trans-generational and remained up to 150 minutes after priming (Rodríguez-Rojas et al. 2019) which was observed in this study. Other studies show that bacteria can retain the priming effects for hours by bimodally switching between virulent and non-virulent subpopulations (Ronin et al. 2017). While some strains of bacteria are known to retain their persistent phenotype long after the stressor has been removed (Miyaue et al. 2018).

As mentioned in section 1.3, biocides have several non-specific targets sites within bacteria (Maillard 2002; Gilbert and McBain 2003; Morente et al. 2013), some of which
are shared with antibiotics (Mangram et al. 1999; Falagas et al. 2005; Li et al. 2006; Wand et al. 2016). It is possible that these shared sites may have been activated by low concentrations of biocides, which would explain the stable cross-resistance to antibiotics observed here.

2.5. Conclusion

At sub-inhibitory concentrations of antimicrobials for extended periods, the chance of developing mutations that can cause reduced susceptibility may be higher compared to when exposed to lethal concentrations (Gullberg et al. 2011; Hughes and Andersson 2012; Andersson and Hughes 2014).

The results from the present study shows that prolonged exposure of bacteria to low concentrations of biocide can cause cross-resistance with antibiotics even in the absence of increased tolerance to the biocide. It is important to understand the origin, evolution and mechanisms of resistance involved in order to tackle the issues presented in this study.

To understand the mechanisms involved, simple identification techniques such as the effects of efflux pump inhibitors will be used to test the presence of efflux mechanisms. Molecular methods such as PCR will be employed to check the presence of and to compare the expression of antibiotic genes between parent and primed strains of bacteria. This will be the focus of following chapters.
3. Chapter Three: Analysis of efflux mechanism as a contributor to adaptive resistance to antibiotics

3.1. Introduction

Bacteria have evolved several protective mechanisms for survival within the ecological niche they share with other antimicrobial-producing microbes, allowing their adaptation to various environmental threats (Poole 2002; Munita and Arias 2016). The most common mechanisms of antimicrobial resistance are discussed below.

3.1.1. Enzymatic inactivation of drugs

The mechanism of action of β-lactam antibiotics against bacteria is the obstruction of cell wall synthesis via the inhibiting of penicillin binding proteins (PBPs) responsible for transglycocylation of peptidoglycan (Tomasz 1979; Munita and Arias 2016). Some bacteria overcome this by producing drug-destroying enzymes such as β-lactamases. These enzymes were first described in the early 1940s, even before the availability of penicillin (Abraham and Chain 1940; D’Costa et al. 2011). Regulated by the blal and the blar1 genes, β-lactamases hydrolyse the amide bond found on the β-lactam ring rendering the drugs ineffective against their targets in bacterial strains such as S. aureus (Hackbarth and Chambers 1993; Bradford 2001; Younas et al. 2018; Sikri et al. 2018).
3.1.2. Effect of cell envelope

The Gram-negative bacterial outer membrane is the first line of defence, creating a natural selective permeable barrier against toxic materials while also allowing sufficient nutrient to enter the cells (Koebnik et al. 2000; Hancock and Brinkman 2002; Fernández and Hancock 2012). Nutrients are taken up by the cells via proteins called porins, porins makes up water filled pores spanning the outer cell membrane (Fernández and Hancock 2012). Porins are classified into two functional groups; the non-specific (general) porins such as OmpF which permits passive diffusion of solutes and antibiotics such as β-lactams (Nikaido et al. 1983) and the specific porins which exacts the Michaelis–Menten kinetics to transport solutes such as maltose, vitamin B12 and nucleosides which otherwise would not able to passively diffuse through the non-specific porin channels (Nikaido, 1992; Schirmer 1998; Denyer and Maillard 2002). Outer membrane porins have been classified in literature according to their structure, selectivity and the way their expressions are regulated (Nikaido and Vaara 1985; Nikaido 2003).

Three types of porins that have been studied in E. coli are; the OmpF (Schabert et al. 1995; Nikaido 2003; Haloi et al. 2018), the OmpC (Nikaido 2003; Kissoyan et al. 2018) and the PhoE porins (Nikaido 2003). The E. coli OmpF porin is made up of 3 OmP proteins with openings on the outer membrane surface for the uptake of up to 600 Da sized hydrophilic molecules, in comparison, the PhoE porin which mostly allows the passage of negatively charged solutes (Denyer and Maillard 2002). In comparison to E. coli classical porins, 6 porins have been associated with the P. aeruginosa outer membrane; the OprF, OprP, OprB, OprD, OprC and OprE (Denyer and Maillard 2002). The OprF, OprP,
OprB and OprD are classified as major porins, the OprC and OprE functions as minor porins (Hancock et al. 1990; Denyer and Maillard 2002).

As previously mentioned, porin proteins significantly contribute to the permeability of the Gram-negative bacterial cell membrane (Fernández and Hancock 2012), therefore any changes such as reduction in size or removal of porin will have a significant effect on the bacteria. For example, changes to porin size have been shown to contribute to the reduced uptake of antibiotics in *P. aeruginosa* (Yoneyama et al. 1995) which was subsequently linked to increased resistance in the bacteria strain (Achouak et al. 2001), especially in OprD and OprD porins where increased resistance to antibiotics such as imipenem and β-lactams were associated with lack of porins (Büscher et al. 1987; Godfrey and Bryan 1987; Denyer and Maillard 2002). Downregulation of outer membrane porins such as *ompF* in *E. coli* makes it difficult for some antibiotics such as the β-lactams, vancomycin and fluoroquinolones, which rely on these protein channels (porins), to enter the bacteria (Robert and Brinkman 2002; Pagès et al. 2008 Hancock).

3.1.3. Active drug removal

Many bacteria remove toxic compounds from their cells with the aid of efflux systems. Studies show that efflux mediated resistance can happen in many antibiotics (Li et al. 1994; Gill et al. 1999; Lin et al. 2002). Active removal of antimicrobials from cells via efflux pumps reduces their intracellular concentrations to levels that are not toxic to bacteria (Kumar and Varela 2013).
A Tet efflux system able to extrude tetracycline from the cytoplasm of *E. coli* was first described in the 1980s (McMurry *et al*. 1980). Several efflux systems have since been described in both Gram-positive and Gram-negative bacteria, while some are specific to certain antibiotics for example the TetA is specific for tetracycline efflux, others have broad antimicrobial specificity resulting in multidrug resistant bacteria (Poole 2005). Five major efflux pump families have so far been described in both Gram-positive and Gram-negative bacteria, examples of each are summarised in Table 3.1 (Webber *et al*. 2003; Van *et al*. 2003; Poole 2005; Van and Lee 2006). The five major groups are as follows; the major facilitator (MFS), the ATP-binding cassette (ABC), the multi-drug and toxic compound extrusion (MATE), the small multidrug resistance (SMR) and the resistance-nodulation-cell division family (RND) (Webber *et al*. 2003; Piddock 2006). The pumps belonging to the ABC family use energy derived from ATP hydrolysis to actively transport antimicrobials and other toxic substances from cells while the other four families are driven by proton motive force from the counter flow of protons (Poole 2007; Sikri *et al*. 2018).

Though the tet efflux pumps are specific for tetracycline efflux, other multidrug efflux pumps such as the *acrAB-tolC* found in enterobacteria and *mexAB-oprM* in *P. aeruginosa* have been shown to have tetracycline has one of their substrates due to their broad antimicrobial specificity, other antibiotics transported by this pump includes β-lactams, fluoroquinolones, macrolides and chloramphenicol (Dean *et al*. 2003; Visalli *et al*. 2003; Amaral *et al*. 2014). Both *acrAB-tolC* and *mexAB-oprM* are members of the RND family of efflux pumps (Dean *et al*. 2003; Visalli *et al*. 2003). Pumps belonging to the RND family
are mostly encoded in the chromosomes of Gram-negative bacteria, with their tripartite structure spanning the width of the whole cell envelope allowing for contact with both the cytoplasm and the external environment (Du et al. 2015; Munita and Arias 2016, Wang et al. 2017). For example, in *E. coli* the *acrAB-tolC* tripartite consists of; the AcrB transporter protein found in the bacterial inner membrane, the AcrA linker protein found in the periplasmic space and the protein channel TolC located in the bacterial outer membrane (Du et al. 2015; Wang et al. 2017). Binding of substrates such as β-lactam antibiotics, tetracycline or disinfectants to AcrB cause a conformational change which requires interactions with AcrA allowing the substrate to be expelled via TolC (Du et al. 2014).

While the RND family of efflux pumps are specific to Gram-negative bacteria, the MFS family of efflux pumps, such as the NorA of *S. aureus*, are more common in Gram-positive bacteria (Handzlik et al. 2013). Some members of this family transport single classes of drugs, for example TetK of *S. aureus* known to transport fluoroquinolones, whereas others such as NorA, QacA (*S. aureus*) and Bmr (*Bacillus subtilis*) transport several drugs such as fluoroquinolones, tetracycline and macrolides (Neyfakh 1992; Neyfakh et al. 1993; Paulsen et al. 1996). Studies show that the *S. aureus* NorA pump can transport disinfectants, mono-cations and fluoroquinolones from cells and may contribute to antiseptic resistance in MRSA (Kaatz et al. 2003).
Table 3.1. Examples of major family of efflux pumps and selected antibiotic substrates

Adapted from Van Bambeke et al. 2003 Poole 2005; Van Bambeke and Lee 2006.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Superfamily</th>
<th>Effluxpump</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-Lactams</td>
</tr>
<tr>
<td>E. coli</td>
<td>RND</td>
<td>AcrAB-TolC</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AcrAD-TolC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AcrEF-TolC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>VegN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SMR</td>
<td>ErmE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABC</td>
<td>MacAB-TolC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MATE</td>
<td>YdeE</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>MFS</td>
<td>Bcr</td>
<td>✓</td>
</tr>
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<td></td>
<td></td>
<td>Dep</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ErmAB-TolC</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TetA-E</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YdeF</td>
<td>✓</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>RND</td>
<td>MexAB-OprM</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MexCD-OprJ</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MexEF-OprN</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MexK-OprM</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MexXY-OprM</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>MFS</td>
<td>TetA, C, E</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ChiA</td>
<td>✓</td>
</tr>
<tr>
<td>S. aureus</td>
<td>MFS</td>
<td>NorA</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NorB</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tet K-L, Te3B</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>ABC</td>
<td>MevA</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MlaA</td>
<td>✓</td>
</tr>
</tbody>
</table>
3.1.4. Efflux pump expression

Efflux pump expression have been known to contribute to resistance in most bacterial strains (Li et al. 1994; Gill et al. 1999; Sikri et al. 2018; de Sousa et al. 2018). Studies show that the inhibition of efflux pumps can reverse bacterial resistance to antibiotics (Poole 2001; Kaatz et al. 2003; Kristiansen et al. 2006; Rao et al. 2018, de Sousa et al. 2018). Efflux pump inhibitors such as phenothiazine, thioxanthene, dopamine receptor antagonists and calmodulin inhibitors have been shown to inhibit efflux pumps in both Gram-negative and Gram-positive bacteria (Cederlund and Mårdh 1993; Kristiansen and Amaral 1997; Poulsen et al. 2018; Martins et al. 2013, de Sousa et al. 2018).

Efflux pump inhibitors have previously been shown to restore the efficacy of antibiotics when used in combination with antibiotics, for example Tohidpour et al. observed increased susceptibility to fluoroquinolones in fluoroquinolone resistant *P. aeruginosa* after the strain was treated with the efflux pump inhibitor phenyl alanine arginyl β-naphtylamide (PAβN) (Tohidpour et al. 2009). Their results indicated that efflux mechanism contributed to the initial resistance observed in the strain, therefore a combination of PaβN and fluoroquinolone made the bacterial susceptible to the antibiotic again (Hirakata et al. 2009; Tohidpour et al. 2009).

Studies show that some efflux pump inhibitors can positively affect the intracellular concentrations of antimicrobials, an example of which can be seen in the work of Leitner et al. (Leitner et al. 2011). In their work, the efflux pump inhibitors tariquidar and
elacridar were used to increase the intracellular levels of ciprofloxacin in ciprofloxacin resistant strain *S. aureus*, they measured the intracellular levels of the antibiotic by monitoring the amount of carbon 14 labelled ciprofloxacin entering the cells (Leitner et al. 2011). Other workers showed that EPIs can also potentiate the effect of antibiotics against resistant strains and prevent further increased resistance (Mahamoud et al. 2007; Zhang and Mah 2008). In a 1992 study by Amaral et al., the MIC of two β-lactams; ceftazidime and ceftriaxone against antibiotic resistant *E. coli* were reversed in the presence of chlorpromazine (CPZ) (Amaral et al. 1992). They observed that the MIC of both antibiotics were reduced from 1.0 mg/l to 0.08 and 0.07 mg/l respectively when the antibiotics were used with sub-inhibitory concentrations CPZ (Amaral et al. 1992).

3.1.5. Mechanisms of efflux pump inhibitors

Several mechanisms of action have been postulated for the effect of EPIs on efflux pumps (Pagès and Amaral 2009), for example they may disrupt the expression of regulatory genes required for efflux pump assembly (Van and Lee 2006) by targeting of small interfering RNA and antisense oligonucleotides in order to selectively hinder the transcription of the genes encoding an efflux pump (Pagès and Amaral 2009). Non-traditional antisense molecules can also be used to block the translation of the gene RNA, an example of which was previously demonstrated in the inhibition of AcrAB efflux pump and can possibly be extended to other efflux pumps (Van and Lee 2006; Pagès and Amaral 2009). Efflux pump inhibitors have been shown to disrupt the components required for efflux pumps assembly (Amaral 2009). For example, globomycin a known
inhibitor of signal peptidase II (SPII) whose function is the removal of lipoprotein signal sequence from exported membrane (Kiho et al. 2004; Tokuda and Matsuyama 2004), can be used to impair the AcrA pump which is an envelope lipoprotein. A phenomenon which was demonstrated by Malléa et al., where sub-inhibitory concentrations of globomycin was able to increase the intercellular concentration of chloramphenicol in multidrug resistant and efflux overexpressing Enterobacter aerogenes (Malléa et al. 2002). Some EPIs such as Phe-Arg-β-naphthylamine (PaβN) do not inhibit efflux pumps but instead compete positively with antibiotics for extrusion (Lomovskaya and Bostian 2006; Lomovskaya et al. 2007; Mahamoud et al. 2007), resulting in the intracellular accumulation of antibiotics to lethal levels (Pagès et al. 2005). Compounds such as carbonyl cyanidem-Chlorophenylhydrazone (CCCP) and potassium cyanide have been shown to cause cell death by reducing energy levels in the Gram-negative bacterial membrane by disrupting proton-motive force (Pagès et al. 2005; Pagès and Amaral 2009). These compounds interfere with ATP hydrolysis in order to disrupt the energy required for pump activity (Nikaido 1994; Mahamoud et al. 2007; Pagès and Amaral 2009; Marino et al. 2013; Rao et al. 2018).

3.1.6. Disadvantages of efflux pump inhibitors

While the actions of EPIs against bacteria are generally shown to be positive, they may come with some disadvantages; for example, their use may induce the expression of alternate efflux pumps that recognizes them as new noxious agents to expel (Li Nikaido 2004; Poole 2005; Piddock 2006; Pagès and Amaral 2009). While the inhibition of
bacterial essential functions, such steps in fatty acid metabolism or targeting AcrA lipoprotein with inhibitors like globomycin are effective at eliminating bacteria (Malléa et al. 2002; Khandekar et al. 2003), bacteria can also recognise and remove them. For example, cerulenin, a known inhibitor of bacteria fatty acid biosynthesis can now be removed by \textit{P. aeruginosa} via MexCD-oprJ efflux pump (Schweizer 1998).

Following exposure of \textit{E. coli}, \textit{S. aureus} and \textit{P. aeruginosa} to low concentrations of hydrogen peroxide (H$_2$O$_2$), chlorhexidine(CHG), benzalkonium chloride (BKC) and glutaraldehyde (GTA) in section 2.2, stable increased tolerance to BKC was observed \textit{P. aeruginosa} exposed to BKC section 2.3.3, cross-resistance to ciprofloxacin and sulphatriad was observed in \textit{P. aeruginosa} exposed GTA section 2.2.3, (\textbf{Figure 2.2}), β-lactam cross-resistance was observed \textit{E. coli} and \textit{S. aureus} exposed to H$_2$O$_2$ and CHG respectively (\textbf{Figure 2.2}). It was therefore important to test for contributory mechanisms to the cross-resistance to antibiotics and tolerance to biocide observed. The work in this chapter will help to understand the mechanisms that were activated by sub-inhibitory concentrations of biocides during the priming process.
3.1.7. Chapter objectives

This chapter will test for some mechanisms that may be contributing to the antibiotic resistance observed in section 2.3.3 by;

- Testing β-lactamase activity with amp-C β-lactamase test, this will be carried out by using Tris/EDTA to permeate antibiotic resistant bacteria cells in order to release β-lactamase enzyme into surrounding media.

- Testing efflux activity with ethidium bromide-agar Cartwheel method, this will be carried out by growing both antibiotic resistant and parent strains bacteria in increasing concentrations of ethidium bromide and comparing fluorescence levels between the strains.

- Looking at the effects of efflux pump inhibitors (EPIs) on the MICs of antibiotics and biocides in wild type and cross-resistant strains of bacteria. This will be carried out using the known efflux pump inhibitors thioridazine (TZ) and chlorpromazine (CPZ). Both TZ and CPZ have been demonstrated to be effective against Gram-positive bacteria, TZ is more effective against Gram-positive bacteria (Martins et al. 2011; Martins et al. 2013). Therefore, TZ and CPZ will be used to treat S. aureus strains and only TZ will be used for both E. coli and P. aeruginosa strains in this study. Antibiotic resistant strains EcH2O2, SaCHG, PaGTA and biocide tolerant strain PaBKC will be grown in concentrations of EPI that does not inhibit their growth along with varying concentrations of the antibiotic they are resistant to and to see if this can be reversed.
3.2. Methods

Bacterial strains, storage and growth conditions

EcH2O2, SaCHG, PaGTA, and PaBKC were produced from priming Escherichia coli ATCC 8739, Staphylococcus aureus ATCC 6538 and P. aeruginosa ATCC 15442 with sub-inhibitory concentrations of biocides as described in section 2.2.9.

Bacterial storage and growth conditions are as detailed in section 2.2.1.

Preparation of inoculum

Inoculum was prepared as previously described in section 2.2.6.

3.2.1. Antibiotics and chemicals

Oxacillin sodium salt, cephalothin sodium salt, cefoxitin sodium salt and ciprofloxacin sodium salt were all obtained and prepared in ranges previously described in section 2.3.3 and 2.3.5. Two efflux pump inhibitors (EPIs); thioridazine hydrochloride (TZ) and chlorpromazine hydrochloride (CPZ) purchased from Sigma-Aldrich, Gillingham United Kingdom were used to test the efflux activity of antibiotic resistant strains. thioridazine hydrochloride (5 g) was stored at room temperature, stock solutions were prepared by dissolving in sterile distilled water, concentration range used was 1-500 mg/l, and chlorpromazine hydrochloride (5 g) was stored at room temperature, stock solutions
were prepared by dissolving in sterile distilled water, concentration range used was 4 - 256 mg/l.

Ethidium bromide (EtBr) (10 mg/ml) which was used for the EtBr Cartwheel method was purchased from Sigma-Aldrich, Gillingham United Kingdom and was stored in a cupboard away from sunlight. Stock solution was prepared daily in sterile distilled water and container was wrapped in foil until use, concentration range used was 0 - 2.5 mg/l. Efflux pump inhibitors and ethidium bromide were all obtained from Sigma-Aldrich, Gillingham United Kingdom.

One litre 10mM Tris, 1 mM EDTA pH 8.0, which was used for β-lactamase test was obtained from Thermo Fisher Scientific.

Sodium chloride (500 g) which was also used for β-lactamase test was purchased from Oxoid LTD Basingstoke Hampshire England was used to prepare saline solution. The solution was prepared by dissolving 8.5 g of sodium chloride in distilled water and was dispensed into universal glass bottles, the bottles were autoclaved at 121°C, 15 psi for 15 minutes, after which they were cooled and stored at room temperature.

3.2.2. Preparation of antibiotic solutions for minimum inhibitory concentration experiments

Antibiotic working solutions were prepared in Mueller-Hinton broth as previously described in section 2.2.5.
3.2.3. AmpC β-lactamase test

For this test, 0.5 mM Tris/EDTA (Fisher scientific) and saline were used to permeabilise EcH2O2 and SaCHG cells to release β-lactamase into the surrounding media.

Sterile filter paper discs were soaked with 20 µl of an EDTA/saline mixture (in a 1:1 ratio), in a sterile petri dish with lid, the discs were left to dry at room temperature then stored at 4°C. Mueller Hinton agar plate was inoculated with susceptible parent strains with inoculum prepared following EUCAST guidelines previously described in section 2.2.6. Two EDTA/saline impregnated filter discs (AmpC discs) were rehydrated with 20 µl saline before adding several colonies of EcH2O2 (β-lactam resistant strain). A 10-µg ampicillin disc purchased from Mast Group Ltd, Merseyside UK, was placed on the inoculated Mueller Hinton agar plate, the inoculated AmpC discs were placed (inoculated face down) on the Mueller Hinton plate either side of the ampicillin disc. Negative control plate was also prepared alongside susceptible strains on AmpC discs. Plates were covered, inverted and incubated at 37°C for 24 hours.

After 24 hours plates were examined for flattening of the zone of inhibition, an example of a positive result is shown in Figure 3.1. The presence of which will indicate enzymatic inactivation of ampicillin. A lack of indentation would indicate a negative outcome. Tests were repeated three times.

The method was also adapted for oxacillin resistant strain SaCHG using 5 µg oxacillin disc.
Figure 3.1. A typical positive ampC β-lactamase result, showing enzymatic inactivation of cefoxitin. Adapted from Black and Thomas 2005.

Showing an example of a positive ampC β-lactamase result in *E. coli* strain. Flattening can be observed around both EDTA impregnated discs placed either side of the cefoxitin disc. The release of β-lactamase into the surrounding media has inactivated the antibiotic being released by the disc therefore preventing the zone of inhibition from spreading.
3.2.4. Preparation ethidium bromide agar plates for testing efflux activity

To test if efflux mechanisms were activated in the antibiotic cross resistance observed in section 2.3.3 and 2.3.5, tryptone-soya agar plates were prepared with varying concentrations of ethidium bromide (EtBr) (0, 0.5, 1 and 2 mg/l). For each bacterial strain tested, two sets of agar plates were prepared (2 plates per concentration). The plates were prepared on the same day they were to be used. Tryptone soya agar (TSA) and was prepared by suspending agar powder 40 g in one litre of distilled water, the agar was melted using a microwave oven. Melted agar (100 ml) was dispensed into glass reagent bottles and autoclaved at 121°C, 15 psi for 15 minutes, after which they were cooled to approximately 50°C. The required amount of molten agar corresponding to the volume of EtBr concentration required was removed with a sterile pipette and replaced with required volume of EtBr. For example, to prepare 100 ml of 2 mg/l concentration of EtBr-agar from 10 mg/l EtBr, 20 µl of molten TSA was removed from the bottle and replaced with 20 µl EtBr. The molten TSA/EtBr was thoroughly mixed and poured into sterile petri dishes, and allowed to set, the plates were stored away from light (Martins et al. 2011; Martins et al. 2013).

Inoculation of ethidium bromide plates for ethidium bromide cartwheel method
The Prepared ethidium bromide agar plates were divided into sections to form a cartwheel pattern, illustrated in Figure 3.2. (Martins et al. 2011; Martins et al. 2013). The adjusted bacterial cultures were inoculated on the plates along the lines, starting from the centre of the plate to the margin. The inoculated plates were covered with foil and incubated at 37°C for 16 hours, after which they were viewed under a UV light. The levels of florescence was measured and recorded in a UV box. Tests were repeated three times.

![Diagram illustrating the EtBr-agar Cartwheel method, adapted from (Martins et al. 2011; Martins et al. 2013)](image)

**Figure 3.2. Diagram illustrating the EtBr-agar Cartwheel method, adapted from** (Martins et al. 2011; Martins et al. 2013)

Diagram illustrating efflux test for bacterial strains using the EtBr-agar Cartwheel method. Antibiotic resistant and wild type bacteria were inoculated on individual TSA plates with varying concentrations of EtBr. The plates were incubated at 37°C for 16 hours. After incubation, fluorescence was observed under ultraviolet light and recorded. Strains that showed lower or no fluorescence indicates efflux activity and strains that shows higher fluorescence indicates no efflux activity.
3.2.5. Establishing MIC of efflux pump inhibitors TZ and CPZ

To establish the concentrations of efflux pump inhibitors that will not inhibit bacterial growth, their minimum inhibitory concentrations (MIC) were tested. The MICs of Efflux pump inhibitor TZ against antibiotic resistant strains EcH2O2, SaCHG, PaGTA and PaBKC and parent strains EcATCC, SaATCC and PaATCC were tested, concentration range used was 1.9 to 1000 mg/l. The MIC of CPZ against antibiotic resistant strain SaCHG and parent strain SaATCC was also established and concentration range used was 4 to 256 mg/l. The Mic tests were carried out by following EUCAST guidelines as previously described in section 2.2.7. Tests were carried out in technical and biological triplicates (n=3).

3.2.6. Effect of efflux pump inhibitors

After establishing the MIC of EPIs against antibiotic resistance strains in section 3.2.5, the effect EPIs on antibiotic resistance was tested. Antibiotic resistant strains were grown in the concentrations of antibiotics they were resistant to along with the concentrations of EPI that does not inhibit their growth. 40 mg/l TZ was used to block EcH2O2 with cephalothin concentration range of 1 to 64 mg/l. 15 mg/l TZ was used to block SaCHG with oxacillin concentration range of 0.0625 to 8 mg/l. 250 mg/l TZ was used to block PaGTA in ciprofloxacin concentration range of 0.0625 to 8 mg/l and PaBKC with BKC concentration range of 16 to 128 mg/l. 20 mg/l CPZ was used to block SaCHG.
The concentrations of EPI used to block each antibiotic resistant strain is shown in Table 3.2.

The Inoculum of primed strains was prepared and standardised in Mueller Hinton broth as previous described in section 2.2.6. The required concentration of efflux pump inhibitor (EPI) was added to the standardised inoculum and thoroughly mixed. Fifty microlitres of the standardised inoculum containing EPI was dispensed into each well of a 96-well plate already containing 50 µl of the required concentrations of antibiotics, to give a final inoculum concentration of 5 x10^5 CFU/ml. Fifty microlites of the standardised inoculum-EPI was added to 50 µl of broth, and 100 µl of broth and inoculum was dispensed into two wells of the 96-well plate as positive and negative control respectively. The inoculated 96-well plate was incubated at 37°C for 24 hours. The lowest concentration of EPI that prevented bacteria growth after 24 hours of incubation was used to establish the MIC. Minimum inhibitory concentration tests were carried out on three separate occasions, with three technical repeats. Minimum inhibitory concentrations of the antibiotics without EPIs were also done at the same time for comparison. The experiments carried out in technical and biological triplicates (n=3).
Table 3.2. Concentrations of efflux pump inhibitor used for each strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>TZ (mg/l)</th>
<th>CPZ (mg/l)</th>
<th>Antibiotic/ biocide</th>
<th>Antibiotic concentration range (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcH2O2</td>
<td>40</td>
<td>-</td>
<td>Cephalothin</td>
<td>1 to 64</td>
</tr>
<tr>
<td>SaCHG</td>
<td>15</td>
<td>20</td>
<td>Oxacillin</td>
<td>0.0625 to 8</td>
</tr>
<tr>
<td>PaGTA</td>
<td>250</td>
<td>-</td>
<td>Ciprofloxacin</td>
<td>0.0625 to 8</td>
</tr>
<tr>
<td>PaBKC</td>
<td>250</td>
<td>-</td>
<td>BKC</td>
<td>16 to 128</td>
</tr>
</tbody>
</table>

- = not tested.
3.3. Results

3.3.1. AmpC β-lactamase test

Following the observed stable β-lactam resistance in EcH2O2 to cephalothin, cefoxitin and ampicillin and in SaCHG to oxacillin, it was necessary to test if β-lactamase contributed to the increased resistance, as β-lactamase is known to be a large contributor to β-lactam resistance in bacteria (Black and Thomas 2005). Cells were grown at 37°C along with discs impregnated with 0.5 mM EDTA. After 24 hours of incubation, plates were examined for indentation or flattening around the zones of inhibition. The results of the β-lactamase test for EcATCC, EcH2O2, SaATCC and SaCHG are shown in Figure 3.3. There was no flattening around the ampicillin zone of inhibition in negative control EcATCC, an indication that β-lactamase was not being released into the surrounding agar, the same observation was made for EcH2O2 (b). Both SaATCC (c) and SaCHG (d) did not show any flattening around the oxacillin zone of inhibition.

There was however observed growth of EcH2O2 within the zone of inhibition made by ampicillin disc (Figure 3.3 b) which is indicated by arrows. The same was observed of SaCHG around the zone of inhibition made by the oxacillin (c) also indicated by arrows. These observations further confirm the antibiotic cross-resistance previously observed in section 2.3.3 and 2.3.5.
To test the effect of β-lactamase, filter discs impregnated with EDTA was placed on TSA plates spread with either β-lactam resistant EcH2O2 or SaCHG and grown at 37°C for 24 hours in the presence of either ampicillin or oxacillin antibiotic discs, in order to permeabilise the membranes of resistant strains to release β-lactamase enzyme into the surrounding media. (a) negative control EcATCC show no enzymatic inactivation of ampicillin (b) test EcH2O2 show no enzymatic inactivation of ampicillin, however growth of EcH2O2 was observed around the ampicillin disc, (c) negative control SaATCC show no flattening around impregnated disc and (d) test SaCHG show no flattering around impregnated discs, however SaCHG was observed growing around the oxacillin disc. n=3.

**Figure 3.3. AmpC β-lactamase test for EcH2O2 and SaCHG**
3.3.2. Ethidium bromide agar (Cartwheel method) of assessing efflux.

To test if efflux mechanisms were involved in the cross-resistance to antibiotics observed in EcH2O2, SaCHG and PaGTA, strains were grown for 16 hours at 37°C, in increasing concentrations of ethidium bromide after which plates were observed under ultraviolet light to compare fluorescence levels. There was no observable difference in the fluorescence levels of both EcATCC and EcH2O2 strains. Results not shown as photographs taken did not transfer well. The results for SaCHG, PaGTA and PaGTA were inconclusive because the strains fluorescence on their own even without ethidium bromide, therefore positive and negative results were not distinguishable (data not shown).

3.3.3. Establishing MIC of efflux pump inhibitors

To test the susceptibility of the antibiotic resistant strains of bacteria against efflux pump inhibitors, the minimum inhibitory concentrations of TZ and CPZ against the strains were established, following the method previously described in section 2.2.7. The MIC was tested in order to know the concentrations of EPI that will not inhibit EcH2O2, SaCHG, PaGTA PaBKC using wild type strains EcATCC, SaATCC and PaATCC as negative controls. The results are shown in Table 3.3. The established MIC of TZ against EcH2O2 was at a higher level of 128 mg/l compared to SaCHG which was 35 mg/l. The MIC of TZ against PaGTA and PaBKC was at a much higher level of 500 mg/l. The established MIC of CPZ against SaCHG was 64 mg/l which was much higher than that of TZ for the same strain.
Table 3.3. Minimum inhibitory concentrations of efflux pump inhibitors

<table>
<thead>
<tr>
<th>Strain</th>
<th>TZ (mg/l)</th>
<th>CPZ (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcH2O2</td>
<td>128</td>
<td>-</td>
</tr>
<tr>
<td>EcATCC</td>
<td>128</td>
<td>-</td>
</tr>
<tr>
<td>SaCHG</td>
<td>35</td>
<td>64</td>
</tr>
<tr>
<td>SaATCC</td>
<td>35</td>
<td>64</td>
</tr>
<tr>
<td>PaGTA</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td>PaATCC</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td>PaBKC</td>
<td>500</td>
<td>-</td>
</tr>
</tbody>
</table>

To establish the concentration of efflux pump inhibitors that would not inhibit parent and antibiotic resistant strains. The MIC results are shown in Table 3.3. The MIC tests were carried out in biological and technical triplicates on three separate occasions =3.

**Key:**

- EcH2O2 - hydrogen peroxide primed *E. coli*
- EcATCC - Parent strain *E. coli*
- SaCHG - chlorhexidine primed *S. aureus*
- SaATCC - parent strain *S. aureus*
- PaGTA - glutaraldehyde primed *P. aeruginosa*
- PaATCC - parent strain *P. aeruginosa*
- PaBKC - benzalkonium chloride primed *P. aeruginosa*
- TZ - thioridazine
- CPZ - chlorpromazine
- - not tested.
3.3.4. Effect of efflux pump inhibitors on bacterial resistance

To see if efflux pump inhibitors would reverse the MIC of antibiotic resistant strains EcH2O2, SaCHG and PaGTA, they were grown in the presence of the concentrations of antibiotic they were resistant to along with sub-inhibitory concentrations of selected efflux pump inhibitors. The effect of efflux pump inhibitor was also tested on benzalkonium tolerant strain PaBKC in the presence the biocide.

3.3.5. The effect of TZ on the resistance of EcH2O2 to antibiotics

The MIC of cephalothin and cefoxitin in the presence of 40 mg/l TZ is summarized in Table 3.4. The established increased MIC of cephalothin and cefoxitin against antibiotic resistant strain EcH2O2 was 32 mg/l and 16 mg/l respectively. In the presence of 40 mg/l TZ, the MIC of cephalothin was reduced by four-folds, from 32 mg/l to 8 mg/l. The MIC against cefoxitin was also reduced by two-fold, from 16 mg/l to 8 mg/l in the presence of 40 mg/l TZ.

Table 3.4. The effect of TZ on the resistance of EcH2O2 to antibiotics

<table>
<thead>
<tr>
<th>Strain</th>
<th>TZ* (mg/l)</th>
<th>MIC (mg/l), mode n =3</th>
<th>cephalothin</th>
<th>cephalothin+ TZ*</th>
<th>cefoxitin</th>
<th>cefoxitin + TZ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcH2O2</td>
<td>40</td>
<td>32</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

TZ, thioridazine
3.3.6. The effect of TZ and CPZ on SaCHG resistance to oxacillin

The effects 15 mg/l TZ and 20 mg/l CPZ on oxacillin resistant strain SaCHG is summarized in Table 3.5. The MIC of oxacillin against antibiotic resistant strain SaCHG was 2 mg/l, this MIC was however reduced by eight-fold to 0.25 mg/l in the presence of 15 mg/l TZ, which was much more effective than 20 mg/l CPZ which only reduced the MIC by four-fold to 0.5 mg/l.

Table 3.5. The effect of TZ and CPZ on the resistance of SACHG

<table>
<thead>
<tr>
<th>Strain</th>
<th>TZ* (mg/l)</th>
<th>CPZ* (mg/l)</th>
<th>Oxacillin</th>
<th>oxacillin + TZ*</th>
<th>oxacillin + CPZ*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SaCHG</td>
<td>15</td>
<td>20</td>
<td>2</td>
<td>0.25</td>
<td>0.5</td>
</tr>
</tbody>
</table>

CPZ, chlorpromazine. TZ, thioridazine
3.3.7. The effect of TZ on PaGTA resistance to ciprofloxacin

Following stable increased 8-fold tolerance (from 0.125 mg/l to 1 mg/l) of PaGTA to ciprofloxacin after exposure to sub-inhibitory concentrations of the glutaraldehyde (GTA) in chapter 2, we wanted to see the efflux pump inhibitor TZ could reverse its to its MIC to 0.125 mg/l. The effect of TZ on the increased resistance of PaGTA to ciprofloxacin could not be determined due to turbidity of the compound at such high concentration of 250 mg/l. However, reduced growth was observed in wells containing both TZ at 250 mg/l and ciprofloxacin at 0.125 mg/l, when bacterial culture from MIC wells were spread onto tryptone soya agar plates and incubated at 37°C for 24 hours, compared the culture from wells with either ciprofloxacin or TZ. Figure 3.4. (a) culture from wells with 250 mg/l TZ alone, there was heavy bacterial growth on the TSA plate (b) culture from wells with 0.125 mg/l ciprofloxacin alone, heavy growth was also observed and (c) however when culture from wells containing 0.125 mg/l ciprofloxacin plus 250 mg/l TZ where observed, there was significant growth reduction in PaGTA.
The effect of 250 mg/l thioridazine on ciprofloxacin resistance

The effect of 250 mg/l TZ on ciprofloxacin resistant strain PaGTA was not easily observed in the 96 well plate because of the turbidity of the TZ when used at such a high concentration. Reduced growth in PaGTA was however observed when culture from turbid wells were spread onto TSA plates and incubated at 37°C for 24 hours then compared to wells with either ciprofloxacin or TZ alone. (a) at 250 mg/l TZ alone, the isolates were too numerous to count, (b) at 0.125 mg/l ciprofloxacin, the isolates were also too numerous to count and (c) at 0.125 mg/l ciprofloxacin plus 250 mg/l TZ, reduced bacterial growth was observed.
3.3.8. The effect of TZ on PaBKC tolerance to benzalkonium chloride

Following stable increased two-fold tolerance (from 64 mg/l to 128 mg/l) of PaBKC to BKC after exposure to sub-inhibitory concentrations of the biocide in section 2.3.3, it was important to know if efflux mechanism could have contributed to the increased tolerance observed. The efflux pump inhibitor TZ was used to test the effect of efflux, however, the effect of TZ on the resistance of PaBKC to BKC could not be determined with the MIC method due to turbidity of the compound at such high concentration of 250 mg/l. To see if there was a change in bacterial growth, culture from MIC wells were spread onto TSA plates and incubated at 37 °C for a further 24 hours, after which they were observed for bacterial growth showed. Figure 3.4 (a) at 250 mg/l TZ, heavy bacterial growth was observed, (b) at 64 mg/l BKC alone there was also heavy bacterial growth seen, (c) however culture from wells containing both 64 mg/l BKC and 250 mg/l TZ, showed no bacterial colonies growing on the TSA plate.
The effect of TZ on the increased tolerance of PaBKC to BKC could not be determined however, reduced growth in PaBKC was observed in the presence of TZ and BKC when turbid wells were spread onto TSA and incubated at 37°C for 24 hours compared to wells with either BKC or TZ alone at the same concentrations. (a) at 250 mg/l TZ, heavy growth of PaBKC was observed, (b) at 64 mg/l BKC alone, heavy bacterial growth was observed and (c) at 64 mg/l of BKC plus 250 mg/l TZ, no bacterial growth was observed.

Figure 3.5. The effect of 250 mg/l thioridazine on BKC tolerance
3.4. Discussion

Following the cross-resistance to antibiotics observed in ECH2O2, SACHG, PAGTA in section 2.3.3 and the increased two-fold tolerance of PaBKC to BKC in section 2.3.1, it was important to test for contributory mechanism to the cross-resistance and tolerance observed. The presence of β-lactamase was tested in β-lactam resistant strains EcH2O2 and SaCHG and the effect of efflux mechanism was tested in all primed strains showing either antibiotic resistance or biocidal tolerance.

3.4.1. β-lactamase test

*Escherichia coli* is an important pathogen which is often implicated in serious infections in both the community and hospital environments and β-Lactam antibiotics such as cephalothin and cefoxitin are often employed in the treatment of this pathogen (Black et al. 2005). In Gram-negative bacteria, resistance to β-Lactam antibiotics is often as a result of β-lactamase production (Livermore 2003; Black et al. 2005). Plasmid mediated AmpC β-Lactamases have a broad antibiotic profile that includes the penicillins and cephalosporin and have been linked to multiple antibiotic resistance (Bradford et al. 1997; Philippon et al. 2002). *Escherichia coli* and *Klebsiella pneumoniae* producing plasmid mediated AmpC β-lactamases have previously been implicated in nosocomial outbreaks (M'Zali et al. 1997, Nadjar et al. 2000; Rice et al. 1990; Papanicolaou et al. 1990). Resistance to most cephalosporins is often as a result of the AmpC phenotype which may result from upregulation of an already present chromosomal ampC gene or an acquired ampC from other Gram-negative bacteria (Forward et al. 2001).
The AmpC disc test is based on the permeabilising effect of Tris-EDTA (Leive 1965) on bacteria cells to release β-lactamase into their external environment (Black et al. 2005). Flattening or indentation of the zone of inhibition would have indicated inactivation of ampicillin by β-Lactamase enzyme however, the results in Figure 3.3. suggests that β-Lactamase enzyme was not being released onto the lawn of bacteria when compared to the positive test previously performed by Black et al. (2005). While the ampC test is cheap and useful for the detection of plasmid associated β-Lactamase enzyme, it may not detect chromosomally encoded AmpC β-lactamase (Black et al. 2005).

The present data indicate that there may be other mechanisms involved in the cross-resistance to β-Lactam antibiotics observed in this study.

3.4.2. Ethidium bromide-agar (Cartwheel) method.

The ethidium bromide-agar (Cartwheel) method is a simple, instrument-free method of detecting multi-drug efflux activity in bacteria (Martins et al. 2011; Martins et al. 2013). The method relies on the ability of Gram-positive and Gram-positive bacteria to pump out the fluorescent molecule, ethidium bromide (a substrate of most efflux pumps). A strain showing fluorescent or increased fluorescent growth would indicate no efflux activity since they would have retained the EtBr. Compared to a strain showing no or less fluorescent growth at the same concentration of EtBr an indication that the EtBr has been removed from the cells via efflux mechanism (Martins et al. 2013).

Due to the cross resistance to antibiotic observed in section 2.3.3 and 2.3.5, we used this method to test if efflux mechanism could be involved. While this method has
previously been used to detect multidrug efflux activities in other studies (Amaral et al. 2014; Martins et al. 2011), this was however not observed in the present study. While cartwheel method is a quick and cheap method for testing the effect efflux mechanisms, the results may not always be conclusive, therefore other confirmatory tests such as the efflux pump inhibitors can be used to confirm the results obtained. In some strains such as in *P. aeruginosa* ATCC 1544 which already produces fluorescence, the results may be difficult to analyze, it is therefore not always reliable.

3.4.3. Effect of efflux pump inhibitors

Antibiotic resistant strains ECH2O2, PAGTA, SACHG and biocide tolerant strain PABKC were grown in the presence of the antibiotics or biocide they were resistant or tolerant to, plus sub-inhibitory concentrations of selected efflux pump inhibitor. This was carried out in order to understand if efflux was involved in the cross-resistance of ECH2O2, PAGTA, SACHG to antibiotics and the increased tolerance of PaBKC to benzalkonium chloride following priming with biocides. Phenothiazines such a TZ and CPZ have been shown to potentiate the effect of antimicrobials against bacteria (Amaral et al. 1992; Kristiansen and Amaral 1997; Cohn and Rudzienski 1997; Wainwright et al. 1998; Amaral and Kristiansen 2001; Viveiros and Amaral 2001), eliminate antibiotic resistant plasmids (Molnar et al. 1982; Evdokimova et al. 1997; Radhakrishnan et al. 1999) and inhibit bacterial efflux pumps (Molnar et al. 1997; Nacsa et al. 1998; Viveiros and Amaral 2001; Viveiros et al. 2002; Ordway et al. 2003; Kristiansen et al. 2006; Costa et al. 2013; Machado et al. 2018).
In the present study both TZ and CPZ have been used as efflux pump inhibitors, TZ was used to block EcH2O2, SaCHG, PaGTA and PaBKC, while CPZ was used to block SaCHG only. Chlorpromazine is the most studied of the two, but they both have the same antimicrobial properties against efflux and phagocytosis (Crowle et al. 1992; Amaral and Kristiansen 2001; Ordway et al 2002; Ordway et al. 2003; Machado et al. 2018). The effect of TZ at sub-inhibitory concentration was tested on EcH2O2 in the presence of varying concentrations of cephalothin and cefoxitin. TZ greatly increased the susceptibility of EcH2O2 to cephalothin and cefoxitin Table 3.4, suggesting that efflux mechanisms may be involved in the cross-resistance observed in this study. Our results agree with the previous findings of Amaral et al. (1992) where CPZ, another phenothiazine with the same properties as TZ, reduced the MIC of ceftazidime and ceftriaxone against E. coli, from 1.0 mg/l with both drugs alone to 0.08 and 0.07 mg/l in the presence of CPZ respectively (Amaral et al. 1992). While their study hypothesised that EPI action promoted increased penetration of the antibiotics to allow easy access to penicillin binding proteins (PBPs) (Amaral 1992), other studies show that efflux pump inhibiting compounds such as phenylalanine-arginine β-naphthylamide (PaβN) and naphthylpiperazines compounds; 1-(1-naphthyl) piperazine, 1-(1-naphthyl-methyl)-piperazine (NMP) actually inhibit efflux pumps (Bohnert and Kern 2005; Schumacher et al. 2005; Schuster et al. 2017). The studies show that EPIs are effective in strains of bacteria such as E.coli only when they overexpress efflux pumps such as acrAB and acrEF, but not in efflux pump deficient strains (Bohnert and Kern 2005; Schumacher et al. 2005; Schuster et al. 2017).
When the effect of both TZ and CPZ on MIC levels of oxacillin on SaCHG was evaluated, the results revealed a significant reduction of MIC in the presence of CPZ Table 3.5. These results suggest that efflux mechanisms may be contributing to the cross-resistance observed, furthermore it agrees with the findings of Kristiansen et al. (Kristiansen et al. 2006), where CPZ and TZ at 25 or 50 % their MIC were able to significantly reduce the MIC of oxacillin in resistant strain S. aureus. This was also observed by Costa et al. where TZ reduced the MIC of ciprofloxacin and norfloxacin in fluoroquinolone resistant strain S. aureus (Costa et al. 2013).

When treated with sub-inhibitory concentration of TZ, there was no observable change in MIC in PaGTA to both sulphatriad and ciprofloxacin, due to solubility issues and resulting turbidity. However, a reduction in growth levels was observed when wells were sub-cultured on agar in the presence of TZ (250 mg/l) and ciprofloxacin (0.125 mg/l) compared to when cultured with either ciprofloxacin or TZ alone Figure 3.4. Although this did not show whether TZ reduced the MIC of both sulphatriad and ciprofloxacin, it did appear to affect the growth levels of the bacteria, an indication that efflux mechanisms may be involved in the cross-resistance observed here, which warrants further investigation.

Although efflux contributes highly to the resistance in P. aeruginosa, reduced influx or impermeability has also been shown to contribute (Li et al. 2000), for example mutation in or loss of oprD a transmembrane porin is shown to be significant in the resistance of carbapenems (Bradford et al. 1999). The data from this present work suggests that although efflux may be a contributor to the observed cross-resistance to ciprofloxacin
and sulphatriad, there may be other factors such as impermeability and drug target alteration at play, hence the incomplete reversal of MIC by the EPI TZ. This will be investigated further.

The growth of PaBKC was reduced in the presence of TZ at 250 mg/l plus BKC at 64 mg/l, compared with 64 mg/l BKC alone Figure 3.5. These results suggest that the increased tolerance to of *P. aeruginosa* observed in section 2.3.2 could be as a result of efflux mechanism being activated. The data agrees with that of Mc Cay et al., where upregulation of *mexEF-oprN*, *mex-AB-oprM* and *mexCD-oprJ*, known for extrusion of unrelated antimicrobial compounds (Lomovskaya et al. 1999) was observed after exposure to sub-inhibitory concentrations of BKC. Their data showed that 32- and 14-fold increased expression levels were observed in *mex-AB-oprM* and *mexCD-oprJ* respectively (Mc Cay et al. 2010).

While some of the results obtained in this chapter, such as the increased susceptibility of EcH2O2 and SaCHG to antibiotics in the presence of efflux pump inhibitors is interesting, the exact mechanism of resistance is still yet to be detected. No questions were answered on the increased resistance of PaGTA to ciprofloxacin, the next line of work will be to investigate specific mechanisms of antibiotic resistance by using both PCR and Realtime PCR to look for mutations in related genes as well as their expression levels.
4. Chapter Four: Mechanisms of adaptive resistance

4.1. Introduction

Antimicrobial resistance is now the leading cause of death worldwide (Erickson et al. 2017), with 10 million antimicrobial-resistance related death predicted by 2050 (O’Neill 2014). Many clinically relevant bacteria have acquired resistance to a number of available antimicrobials and will continue to do so unless new ways are found to tackle the emergence of new mechanism of resistance (Centre for Disease Control and Prevention (US) 2013; Erickson et al. 2017). A major contributing factor in the development of bacterial resistance is their intrinsic nature and ability to evade killing, by adapting survival mechanisms (Fernández et al. 2011). While these adaptive survival mechanisms are usually temporary and non-genetic (Adam et al. 2008), genetic changes may arise from the introduction of heterogenous elements during the adaptation process (Motta and Aldana 2015; Aldana 2015; Erickson et al. 2017). Spontaneous mutations also contribute to bacterial resistance, an example of this was shown in the work of Painter et al. (2015) where exposure of S. aureus to sub-inhibitory concentrations of H₂O₂ caused the emergence of gentamicin resistant small-colony variant strain with increased catalase production which also made the strain less susceptible to the biocide. Mutations linked to DNA double-strand break and repair, which like that of E. coli is error prone, was said to be responsible (Painter et al. 2015).
4.1.2. Bacterial protective response

When bacteria such as *E. coli* is faced with adversity, such as oxidative and redox stress conditions, they may activate protective stress response regulons such as *oxyR* and *soxRS* (Nunoshiba *et al.* 1992; Manchado and Pueyo 2000). *Escherichia coli* for example, can co-ordinate the expression of genes belonging these regulons to suite their immediate need (Neihardt 1987).

The SoxRS as well as OxyR regulons are known to be activated by biocides such as hydrogen peroxide and paraquat (Nunoshiba *et al.* 1992; Chou *et al.* 1993; Aslund *et al.* 1999; Machando *et al.* 2000), both regulons have been shown to control the expression of over 100 oxidative stress response genes (Blanchard *et al.* 2007; Bruce 1991; Blanchard 2012). The *soxRS* regulon controls the expression of multiple antibiotic resistance genes such as the efflux pump gene *acrAB*, and the *micF* gene. The *micF* is an antisense RNA that negatively regulates the expression of *E. coli* outer membrane porin gene *ompF* (Tsaneva and Weiss 1990; Greenberg *et al.* 1991; Manchado and Pueyo 2000).

The *soxRS* regulon is activated via the oxidation of the transcription factor SoxR, which in turn induces the expression of a second transcription factor SoxS (Storz and Imlayt 1999). Studies show that the *soxR* and the *soxS* genes are connected in a head to head arrangement (Amabile-Cuevas and Demple 1991; Wu and Weiss 1991; Wu and Weiss 1992), whereby the *soxR* promoter is found within the intergenic region of the regulon while the *soxS* promoter is placed within the *soxR* gene (Nunoshiba *et al.* 1992). Upon activation by SoxS, *micF* has been shown to repress OmpF, one of the classical porins.
found in *E. coli* which is responsible for the passive uptake of several compounds (Nikaido and Vaara 1987; Chou et al. 1993).

While the mechanisms of antibiotic resistance have been well studied and documented in literature, the mechanisms of biocide tolerance and the parallel resistance mechanisms have been less studied (Russell et al. 1997; Russell 2002a; Russell 2002b; Russell 2003b; Russell 2004; Sheldon 2005). Studies have shown that the reduced effects of any biocide against bacteria (Russell 2002b; Russell 2003b; Maillard 2005; Maillard 2011; Paul et al. 2019) corresponds with the reduced concentration of biocides (Russell 2002a). Therefore the potency of any biocide is controlled by its concentration (Russell and McDonnell 2000; Maillard and Denyer 2009; Rajkowska et al. 2016). In some studies, increased tolerance in bacteria have been observed after exposure to sub-inhibitory concentrations of biocides (Abdelaziz et al. 2019), for example Abdelaziz et al. in their 2019 work showed that exposing *Klebsiella pneumoniae* to sub-inhibitory concentrations of benzalkonium chloride caused increase resistance to several antimicrobial agents such as ampicillin and ciprofloxacin, via increased efflux and membrane depolarization (Abdelaziz et al. 2019).

4.1.3. Changes in gene expression

Biocides can activate the expression of genes that contribute to antibiotic resistance in bacteria, as was shown in the study by Huet et al. (2008) where exposure of bacteria, in this case clinical isolates of *S. aureus* to sub-inhibitory concentrations of biocides and dyes, were able to upregulate the expression of multi-drug efflux pump genes; *mepA*,

129
mdeA, norA and norC and mepA and increased MIC to antimicrobials such as chlorhexidine, benzalkonium chloride and ethidium bromide (Huet et al. 2008). In another study carried out by Bialy et al. (2009) exposure of E. coli and Salmonella enterica serovar Typhimurium to triclosan led to the increased expression of efflux pump genes acrB in E. coli and acrE and tolC in Salmonella enterica Serova Typhimurium (Bailey et al. 2009).

As mentioned in the introductory section 3.1, the first line of defence in Gram-negative bacteria against damage from biocides and other noxious compounds is their double membrane cell envelope, which consists of an outer phospholipid and lipopolysaccharide (Stavenger and Winterhalter 2014; Zgurskaya et al. 2015; Silver 2016). The outer membrane is spanned by water-filled β-barrel channels (porins) which permits the diffusion of hydrophilic compounds (Schulz 2002; Nikaido 2003; Delcour 2003; Masi et al. 2019). There are 3 well studied classical porins found in E. coli encoded by; ompF, ompC and phoE (Pagès et al. 2008; Masi et al. 2019) of which ompF is known to be selected for by sub-inhibitory concentrations of antibiotics (Harder et al. 1981; Masi et al. 2019).

Bacterial adaptive response to biocides may involve regulating membrane permeability which includes reducing the uptake of antimicrobials by down regulating porin expression, increasing expression of efflux pumps or a combination of the two( Nikaido and Vaara 1985; Poole 2002; Nikaido 2003; Piddock 2006; Davin-Regli et al. 2008; Pagès et al. 2008; Masi et al. 2017; Dam et al. 2018). These actions by bacteria may promote matching protections against biocides and unrelated antibiotics (Sidhu et al. 2002;
Maillard 2007). An example of this can be seen in the work of Karatzas et al. (2007) where *Salmonella* sp. adapted with a formaldehyde and glutaraldehyde based quaternary ammonium compound displayed reduced porin expression and increased expression of *acrAB-tolC* efflux pump. The bacteria showed resistance to both the disinfectant and other unrelated antibiotics such as ciprofloxacin and ampicillin (Karatzas et al. 2008). The same was also seen in a previous study carried out by the same group where *S. enterica* was treated with triclosan (Karatzas et al. 2007).

4.1.4. DNA damage and repair in bacteria

While changes in gene expression levels may contribute to antibiotic resistance, other changes that may occur to bacteria during exposure to biocides are gene nucleotide changes due to mutations (Imlay and Linn 1986, Imlay and Linn 1987). In *E. coli* for example, two modes of killing were previously described when the bacteria are treated with H$_2$O$_2$ (Imlay and Linn 1986, Imlay and Linn 1987). Mode one is shown to be activated in the presence of metabolically active cells during H$_2$O$_2$ exposure, while mode two as been shown to happen in the absence of metabolism (Imlay and Linn 1987, Imlay and Linn 1986, Brandi et al. 1989). Low concentrations of H$_2$O$_2$ can activate a protective response from *E. coli*, promoting increased resistance during subsequent exposure (Demple and Halbrook 1983, Christman et al. 1985).

Protective enzymes such as catalases, peroxidases and superoxide dismutase are induced in *E. coli*, *S. aureus*, *P. aeruginosa*, *Salmonella enterica* Serova Typhimurium and other enteric bacteria to prevent oxidative damage caused by reactive oxygen species.
(Richter and Loewen 1981, Finn and Condon 1975, Pomposiello, Demple 2001, Chiang and Schellhorn 2012). Therefore two non-overlapping protective stress responses are activated during *E. coli* exposure to H$_2$O$_2$; the oxyR regulon which upregulates the production of scavengers of reactive oxygen species and the SOS (an error-prone system), which is important in recombinant DNA repair (Hidalgo and Demple 1996, Imlay and Linn 1986, Imlay, Linn 1987, Chiang and Schellhorn 2012, Pomposiello and Demple 2001). Studies show that *E. coli* strains lacking RecA proteins (essential for DNA repair and maintenance), DNA polymerase I, exonuclease III and exonuclease V (required for DNA repair) are more prone to mode one killing, suggesting that DNA is the target for mode one killing by H$_2$O$_2$ (Imlaya and Linn 1987, Imlay and Linn 1988).
4.1.5. Chapter objectives

The work in this chapter will build on the results presented in previous chapters, where antibiotic resistance was observed in EcH2O2, SaCHG and PaGTA after exposure to low concentrations of biocides (Section 2.3.3) and efflux pump inhibitors thioridazine and chlorpromazine was able to reverse β-lactamase cross-resistance of EcH2O2 and SaCHG. The work in this chapter will help identify other possible molecular mechanisms involved. This will be achieved by:

- Confirming the presence of genes encoding selected efflux pumps, porins and their regulators in wild type and antibiotic cross-resistant strains EcH2O2, SaCHG and PaGTA by using designed primers to carry out PCR. The selected genes that will be studied in this chapter will include:

  (i) the *E. coli* soxR, soxS, oxyR and a selection of the antibiotic resistance genes they regulate. The selection of these genes is based on the known effect of low concentration of H2O2 and oxidative stress on the activation of the soxRS and oxyR regulons and the regulatory effects of these regulons on antibiotic resistance genes (Nunoshiba *et al.* 1992; Chou *et al.* 1993; Aslund *et al.* 1999; Machando *et al.* 2000).

  (ii) the *norA* efflux gene which has previously been shown to be activated by chlorhexidine in *S. aureus* and known to cause resistance in the bacteria (Hardy *et al.* 2017), for comparison, the *lmrS* efflux gene which encodes another *S. aureus* multidrug
efflux pump (Floyd et al. 2010) will also be studied. These genes were selected due to the cross resistance to oxacillin observed in Section 2.3.3 after S. aureus was exposed to sub-inhibitory concentrations of the biocide.

(iii) The P. aeruginosa mexL and mexJ genes were selected for this study due to previous biocide related resistance in P. aeruginosa being attributed to active efflux (Chuanchuen et al. 2002; Olivares et al. 2012; Vikram et al. 2015) and the cross-resistance to ciprofloxacin and sulphatriad observed in section 2.3.3 after P. aeruginosa was exposed to sub-inhibitory concentrations of glutaraldehyde.

- Sequencing suspected antibiotic resistance genes to identify presence of mutations which can be introduced by the error prone DNA repair systems in both E. coli and S. aureus to reduce the DNA damage caused by oxidative stress caused by reactive oxygen species (Hidalgo and Demple 1996, Imlay and Linn 1986; 1987; Valderas and 2001; Liu et al. 2005; Chiang and Schellhorn 2012, Pomposiello and Demple 2001). Changes in the S. aureus norA promoter region which can be caused by exposure to chlorhexidine will also be studied (Hardy et al. 2017).

- Comparing the expression levels of efflux and porin genes between parent and primed bacterial strains using quantitative real-time PCR. To see if changes in their expression levels corresponds to changes in nucleotide sequence of regulatory genes like soxRS and the genes they regulate such as ompF and acrAB to see if this links with the antibiotic resistance previously observed.
4.2. Methods

4.2.1. Bacterial strains, storage and growth conditions

*Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 15442, Staphylococcus aureus ATCC 6538, ECH2O2 PAGTA, and SACHG* were stored and grown as previously described in section 2.2.1.

4.2.2. Reagents and chemicals

RNase away spray, SyBR™ safe DNA gel stain in 0.5X TBE, GeneRuler 50 bp DNA ladder ready to use and ultrapure™ agarose were obtained from Thermo Fisher. RNeasy Mini Kit (Qiagen), Tetro cDNA synthesis kit (30 rns) (stored at -20°C), SensiFAST SyBR Lo-Rox kit (stored at -20°C) were obtained from Bioline. Monarch PCR and DNA Cleanup kit (5 µg) and Hot Start High-Fidelity 2X master mix (stored at -20°C) were obtained from New England Biolabs Inc®.
Table 4.1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>TM °C</th>
<th>Direction</th>
</tr>
</thead>
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<tr>
<td>acrA</td>
<td>TGT TCG TGA ATT TAC AGG CG</td>
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<td>Forward</td>
</tr>
<tr>
<td>acrA</td>
<td>TGC CAA CAT GAT GAT AAT GGC G</td>
<td>62</td>
<td>Reverse</td>
</tr>
<tr>
<td>acrB1</td>
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<td>63</td>
<td>Forward</td>
</tr>
<tr>
<td>acrB1</td>
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<td>63</td>
<td>Reverse</td>
</tr>
<tr>
<td>acrB2</td>
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<td>AcrB2</td>
<td>GTGGTGCACCGATGGTAATC</td>
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<td>Reverse</td>
</tr>
<tr>
<td>marA</td>
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<td>58</td>
<td>Forward</td>
</tr>
<tr>
<td>marA</td>
<td>ATT AGT TGG CCA GGA CCT GC</td>
<td>69</td>
<td>Reverse</td>
</tr>
<tr>
<td>marB</td>
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<td>Forward</td>
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<td>marB</td>
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</tr>
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<tr>
<td>oxyR</td>
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<td>Reverse</td>
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<tr>
<td>sodA</td>
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<tr>
<td>sodA</td>
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<td>Reverse</td>
</tr>
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<td>soxR</td>
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<td>Forward</td>
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<td>soxR</td>
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<td>Forward</td>
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<td>soxS</td>
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<td>soxS</td>
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<td>ompF</td>
<td>CAGACACATAAGAGACCCAAACTC</td>
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<td>ompF</td>
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</tr>
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<td>Forward</td>
</tr>
<tr>
<td>norA</td>
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<td>Reverse</td>
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</tr>
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<td>mexI</td>
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</tr>
<tr>
<td>mexJ</td>
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<td>Reverse</td>
</tr>
<tr>
<td>mexL</td>
<td>TGG GAC ATC ATG CAC GAC G</td>
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<td>Forward</td>
</tr>
<tr>
<td>mexL</td>
<td>ATACAG AAC CCA TCA GTC CAG</td>
<td>64</td>
<td>Reverse</td>
</tr>
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</table>
4.2.3. Polymerase chain reaction (PCR)

PCR was carried out in a Labnet multigene™ optimax thermal cycler, using genomic DNA previously extracted as described in section 2.2.13. The below parameters were used unless otherwise stated. Reaction concentrations and volumes are shown in Table 4.2.

**Table 4.2. PCR reaction volume**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volumes (µl)</th>
</tr>
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<tbody>
<tr>
<td>Forward primer (100 pmol/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer (100 pmol/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Genomic DNA (~0.1 µg/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Q5® Hot Start 2X Master Mix</td>
<td>25</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>22</td>
</tr>
</tbody>
</table>

**Cycling parameters for PCR**

- Initial denaturation: 98°C - 30 seconds
- Denaturation: 98°C - 30 seconds
- Annealing: 62 °C -20 seconds
- Extension: 72 °C -20 seconds
- Final extension: 72°C -2 minutes.
4.2.4. Agarose gel electrophoresis

Five microliters of resulting DNA samples were mixed with 6X loading dye (Thermo fisher) and analysed by gel electrophoresis as previously described in section 2.2.16.

**PCR product purification for sequencing**

The monarch PCR and DNA cleanup kit (NEB) was used to purify PCR products following manufacturer’s instructions. DNA concentration was measured using a Nano drop (Nanodrop Lite Spectrophotometer, Thermo Fisher Scientific, Loughborough, UK), after calibrating with 1 µl nuclease free water and sequencing was performed as previously described in sections 2.2.14 and 2.2.17.
Table 4.3. Realtime PCR primers used in this study

<table>
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<th>Primer</th>
<th>Sequence</th>
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</thead>
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<td>acrA</td>
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<td>acrA</td>
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<td>acrB</td>
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</tr>
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4.2.5. RNA extraction

A single bacterial colony from 24-hour tryptone soya agar plates was transferred into 10 ml tryptone soya broth and incubated for at 37°C in a shaker orbital incubator of 3 x g speed for 24 hours. Parent and antibiotic cross-resistant bacteria were grown in in tryptone soya broth containing either concentrations of antibiotics or without. *Escherichia coli* strains EcATCC and EcH2O2 were grown in 4 mg/l cephalothin, *S. aureus* strains SaATCC and SaCHG were grown in oxacillin (0.0625/0.125 mg/l) and *P. aeruginosa* strains PaATCC and PaGTA were grown in 0.125 mg/l ciprofloxacin. Bacteria were incubated at 37°C in a shaker incubator at 3 x g speed. Cells were harvested and stabilized with 2 volumes RNAprotect bacteria reagent, cells were incubated at room temperature for five minutes and pelleted by centrifugation for 10 mins at 5000 x g. Supernatant was decanted, then pellets and resuspended in 100 µl TE buffer containing lysozyme, (1 mg/l for *E. coli*, 7 mg/l for *P. aeruginosa* and 20 mg/l + 20 µl protein kinase k for *S. aureus*), they were incubated at 37°C for 1 hour. After incubation, 350 µl buffer RLT containing 10 µl/ml β-mercaptoethanol was added, mixture was vortexed vigorously (*S. aureus* strains were sonicated for 10 minutes) after which 250 µl 96 % ethanol was added and mixed in by pipetting to lyse cells. Strains were grown in biological triplicates for each experiment.
**RNA purification**

To extract RNA, lysed cells were transferred to labelled Eppendorf tubes and RNA was extracted using RNeasy Mini Kit (Qiagen), following manufacturer instructions. Bound RNA was eluted by adding 30 µl RNase free water to the spin column and centrifuged at 1,000 rpm for one minute. Eluted RNA was collected into 1.5 ml sterile Eppendorf tubes.

Further purification of RNA was carried using Invitrogen DNASE set following manufacturer’s instruction. Cleaned RNA was ready for quantification.

**Nano drop spectrophotometry**

Extracted RNA (1 µl) was quantified in Nanodrop spectrophotometer (Nanodrop Lite Spectrophotometer, Thermo Fisher Scientific, Loughborough) after being calibrated using the same quantity of nuclease free water. This was to assess both quality and quantity of RNA extracted. Absorbance was measured at 260 mm and 280 mm and purity ratio of ~2.0 was accepted as pure. Quantified RNA was stored at -80°C ready for CDNA synthesis.

**cDNA synthesis for Realtime PCR**

To obtain the required concentrations of RNA for quantitative Realtime PCR, RNA values obtained from Nanodrop spectrophotometry was used to determine the concentration (100 ng/µl) of RNA and DEPC water required for cDNA synthesis. cDNA synthesis was carried out following manufacturers instruction using super-mix volumes shown in...
Table 4.4. Super-mix volumes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volumes (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo (dT)</td>
<td>4</td>
</tr>
<tr>
<td>dNTPs mix (10 Mm)</td>
<td>4</td>
</tr>
<tr>
<td>5X RT buffer</td>
<td>16</td>
</tr>
<tr>
<td>RiboSafe RNase inhibitor</td>
<td>4</td>
</tr>
<tr>
<td>Tetro reverse transcriptase</td>
<td>4</td>
</tr>
</tbody>
</table>
Eight microliters of the prepared super-mix were added to RNase free reaction tubes containing quantity of RNA samples and DEPC treated water already calculated. After gentle mixing by pipetting, samples were incubated at 45°C for 30 minutes and reaction was terminated at 85°C (enzyme deactivation) for five minutes. cDNA in tubes were placed on ice for qPCR reaction or stored at 20°C. No reverse transcriptase cDNA samples, where reverse transcriptase was substituted with water was generated alongside.

4.2.6. Quantitative Realtime PCR (qPCR)

SensiFAST™ SYBR® Lo-ROX Kit was used to determine gene expression profile of interest in cDNA synthesized above. 2 µl of cDNA from parent and primed strains were used in this analysis. Primer-pair sequence are shown in Table 4.3 and a complete table with accession numbers are shown in Tables 8.1, 8.2, 8.3 and 8.4 (appendix)

Reactions were carried out in biological and technical triplicates in 96-well optical PCR plates (ThermoFisher), reaction volumes are shown in Table 4.5. No template control (NTC) and no reverse transcriptase control (NTC) were included as negative controls in all gene expression reactions.
### Table 4.5. reagents volumes for real time PCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per test</th>
</tr>
</thead>
<tbody>
<tr>
<td>SensiFAST™ SYBR® Lo-ROX</td>
<td>5 µl</td>
</tr>
<tr>
<td>forward primer (10 µM)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>reverse primer (10 µM)</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>cDNA template (10 ng/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>2.2 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

### Real time PCR cycling parameters

- Initial denaturation: 95°C - 2 minutes
- Denaturation: 95°C - 5 seconds
- Annealing: 65°C - 10 seconds
- Final extension: 72°C

40 Cycles
Quantification of relative gene expression

After each reaction, quantification cycles (Cqs) were calculated with PikoReal software version 2.2 (Thermo Fisher). Zero Cq values in corresponding NTC and NRTC wells indicated no significant genomic DNA contamination, however few Cq values of NRTC four to five cycles higher than that of sample is acceptable.

Pfaffl ΔΔCq method was used to quantify relative gene expression using $2^{\Delta\Delta C_q}$ to calculate expression fold change as follows;

$$\Delta C_q = C_q (\text{Target}) - C_q (\text{Reference})$$

$$\Delta\Delta C_q = \Delta C_q (\text{Treated}) - \Delta C_q (\text{Control})$$

Fold change$= 2^{\Delta\Delta C_q}$

$\Delta C_q$ represents the difference in Cq calculated between target and housekeeping/reference genes (depending on one selected for strain) in each sample and $\Delta\Delta C_q$ is the calculated difference in $\Delta C_q$ values obtained from both treated and control samples. Treated sample is bacteria that was previously primed after being exposed to increasing concentration of biocides with increased tolerance to antibiotics and control sample is parent/wild type bacteria unexposed to biocides. Gene expression levels $> 1$ were considered up-regulated and levels $< 1$ were considered downregulated in treated groups (Pfaffl 2001). IBMS SPSS version 25(Wagner III 2019), paired sample $t$-test was used to calculate significant difference in gene expression between parent and primed strains bacteria.
4.3. Results

4.3.1. Primer design

Following establishment of cross-resistance with antibiotics after exposure to sub-inhibitory concentrations of biocide (chapter 2), primers were designed to identify genes, such as regulatory genes, that may contribute to antibiotic resistance mechanisms.

The complete genome sequence of the strains of interest and that of the gene of interest were searched and downloaded from the National Center for Biotechnology Information (NCBI) website, [https://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/). The gene sequence with 200 bp down- and upstream was selected and used to design primers. Forward and reverse primer sequences were designed on either side of the gene of interest (Table 4.1). The OligoAnalyzer tool on [https://www.idtdna.com/pages](https://www.idtdna.com/pages) was used to check for melting temperature, GC content, hairpin formation, self-dimer and hetero-dimer. NCBI Primer BLAST tool was used to verify the specificity of each forward and reverse primers, so they won’t bind to other genomic regions (accession number of primers used are shown in Table 8.1 - 8.4 (appendix)).
4.3.2. Polymerase chain reaction (PCR) and gene sequence analysis

Following observed cross-resistance of primed strains to antibiotics, biochemical analysis and 16s rRNA sequencing were used to identify and confirm strains in section 2.3.7. The effect of efflux pump inhibitors on the antibiotic susceptibility of resistant strains in sections 3.3.5 and 3.3.6 indicated efflux mechanisms may be involved the antibiotic cross-resistance observed. It was therefore important to see if the increase in resistance could be linked to changes in nucleotide sequences and expression levels of suspected antibiotic-resistant genes. The primers designed and used for PCR were between 25-30 base pairs in length with a GC content is not more than 50 %, GC clamped at the 3’ end (to enhance annealing) and Tm of 57 to 62°C.

The mutational effects if sub-inhibitory concentrations of hydrogen peroxide on E. coli stress response and regulatory genes

Exposure of E. coli to low concentrations of H₂O₂ caused cross-resistance to β-lactam antibiotics in section 2.3.3, since H₂O₂ is known to activate the response of protective regulons such as the soxRS which in-turn triggers the upregulation of antibiotic resistant genes, it was important to know if these genes were present in both parent and wild type strains. Since H₂O₂ is also known to cause DNA damage in E. coli it was necessary to know if any mutations occurred during the adaptations process and if this may have contributed to the increase antibiotic resistance observed. Polymerase chain reaction was used to identify the presence of selected gene of interest, Gene specific primers were used for each PCR reaction, products were separated by gel electrophoresis on 1
% agarose gel. Figure 4.1 (a, b and c) EcATCC and EcH2O2, shows primers used in the reactions identified bands corresponding with expected band sizes.

To see if any changes to nucleotide and amino acid sequence occurred during the H₂O₂ adaptation process, primers specific for soxRS and oxyR genes along with acrA, acrB, ompF, micF, sodA, oprD, marA, marB, marC and marR were designed and used to carry out PCR. PCR products were purified and sequenced, after which sequences were analysed using NCBI BLAST in order to see if there were changes to nucleotide and amino acid sequences between parents and antibiotic cross-resistant strains. Of the thirteen EcATCC and EcH2O2 genes identified by PCR and analysed by sequencing, only acrA showed changes in nucleotide sequence. There was a single cytosine to thymine change in codon 255 in acrA coding region Figure 4.1 (d), corresponding with a proline to serine (Pro-255 to Ser-255) change in Figure 4.1 (e). This observed change can affect the structure, folding and therefore the overall function of the protein, depending on the original function of the affected region.
Gene-specific primers were used for each PCR reaction; PCR products were separated by gel electrophoresis on 1% agarose gels. The standard of the molecular weight ladder, 50 bp (lane 1) are shown on the left. Primers used for the reactions corresponds to; (a) EcATCC and (b) EcH2O2, Lane 1, 50bp ladder, Lane 2, acrA expected size 1450 bp, Lane 3, marA expected size 580 bp, Lane 4, marR expected size 740 bp, Lane 5, marC expected size 890 bp, Lane 6, oxyR expected size 1111 bp, Lane 7, sodA expected size 713 bp, Lane 8, soxR expected size 773 bp, Lane 9, soxS expected size 347 bp. (c)EcATCC and EcH2O2 lane 2 and 3 acrB expected size 2067 bp, lane 4 and 5 marB expected size 429 bp, lane 6 and 7 oprD expected size 1684 bp, lane 8 and 9 ompF expected size 1442bp and lane 10 and 11 acrB2 expected size 1802bp, (d) and (e) Purified acrA PCR gene products were sequenced and analysed with NCBI BLAST, a single cytosine to thymine change in codon 255 in acrA , corresponding with a proline to serine (Pro-255 to Ser-255) change.
The mutational effects if sub-inhibitory concentrations of chlorhexidine on *S. aureus* norA and ImrS efflux genes.

Polymerase chain reaction (PCR) was used to identify efflux genes *norA* and *ImrS* in SaATCC and SaCHG. The *norA* efflux gene was selected because it has previously been shown to be activated by chlorhexidine in *S. aureus* which is shown to cause resistance in the bacteria at low concentrations (Hardy *et al.* 2017) and the cross-resistance to oxacillin observed in Section 2.3.3 after *S. aureus* was exposed to sub-inhibitory concentrations of the biocide.

Gene specific primers were used for each PCR reaction, products were separated by gel electrophoresis on 1% agarose gel. **Figure 4.2 (a)** shows primers bound to; *ImrS*, expected size 1684 base pairs lane 2 and 3 lane and *norA*, expected size 1530 base pairs, lane 4 and 5.

The *norA* and *ImrS* PCR product obtained for *norA* and *ImrS* were sequenced and analysed using NCBI BLAST to identify any changes in nucleotide sequence. A single adenine to thymine change at codon 313 was identified in SaCHG coding region of *norA* sequence **Figure 4.2 (a)** which corresponds with isoleucine to phenylalanine change in amino acid sequence **Figure 4.2 (b).**
There were no nucleotide changes in *lmrS* gene sequence (data not shown), *norA* promoter region was also sequenced but there was no nucleotide changes observed

**Figure 8.2** (appendix).

---

**Figure 4.2. PCR and sequence analysis of *norA* and *lmrS* genes**

Gene-specific primers were used for each PCR reaction; PCR products were separated by gel electrophoresis on 1% agarose gels. The standard of the molecular weight ladder 50 bp (lane 1) are shown on the left. (a), Primers used for the reactions corresponds to; Lane 2 SaATCC *lmrS* (expected size 1684 bp), lane 3 SaCHG *lmrS* (expected size 1684 bp), lane 4 SaATCC *norA* (expected size 1530 bp), lane 5 SaCHG *norA* (expected size 1530 bp), Lane 6 50kb DNA ladder. *norA* gene was sequenced and analysed in both SaATCC and SaCHG, (b) single adenine to thymine change in *norA* at codon 313, which corresponds with isoleucine to phenylalanine change (I-313 to F-313) (c) was observed in SaCHG *norA* coding region.
The mutational effects if sub-inhibitory concentrations of glutaraldehyde on *P. aeruginosa* efflux gene *mexJ* and its repressor *mexL*

Following the cross-resistance to ciprofloxacin and sulphatriad in Section 2.3.3 after *P. aeruginosa* was exposed to sub-inhibitory concentrations of glutaraldehyde, gel electrophoresis of PCR product was used to identify the presence of efflux gene *mexJ* and its repressor *mexL* in both PaATCC and PaGTA. These efflux genes were selected due to previous biocide related resistance in *P. aeruginosa* being attributed to active efflux (Chuanchuen *et al.* 2002; Olivares *et al.* 2012; Vikram *et al.* 2015).

Gene specific primers were used for each PCR reaction, products were separated by gel electrophoresis on 1 % agarose gel Figure 4.3. Primers binds to; *mexJ* gene, band fragment matched expected size of 1274 base pair Lane 2 and 3, band fragment matched expected size of 1361 base pair for *oprD* gene lane 6 and 7 and lane 8 and 9 band size matched *mexL* gene expected 785 base pairs was observed. Sequence analysis of *mexJ*, *mexL* and *oprD* genes identified no change in both nucleotide and amino acid sequence (data not shown).
Figure 4.3. PCR of *mexJ* and *mexL* genes

Gene-specific primers were used for each PCR reaction; PCR products were separated by gel electrophoresis on 1 % agarose gels. The standard of the molecular weight ladder 50 bp (lane 1) are shown on the left. Primers used for the reactions corresponds to; Lane 2 and 3 *mexJ* (expected size 1274 bp), lane 6 and 7 *oprD* (expected size 1361 bp) and lane 8 and 9 *mexL* (expected size 785bp).
4.3.3. Quantitative Realtime PCR

**Realtime PCR primers design**

Forward and reverse primers were designed within the gene of interest, parameters include; primer lengths between 18 to 24 bases to yield melting temperature of between 60 to 62°C, GC content of between 50 to 65%. Designed primers were checked for secondary structure formations using the tools described in designing PCR primers. Primers for Realtime PCR are shown in Table 4.3.

**Housekeeping gene selection and validation**

Housekeeping genes are used to normalise the amount of mRNA present in selected genes of interest, before their expression levels are compared in treated and untreated samples using QRTPCR. (Suzuki et al. 2000). Housekeeping genes therefore quantifies and reduces errors that may arise from RNA extraction, RNA quality, cDNA synthesis or may be present in the endogenous controls used (Tichopad et al. 2003, Tichopad et al. 2004; Peters et al. 2004). Housekeeping genes used in this study were selected using previously validated methods (Rocha et al. 2015). GeNorm version 3.5 was used to determine and select stably expressed housekeeping genes or gene pair across sample conditions (Vandesompele et al. 2002). Primers designed for the housekeeping genes are included in Table 4.3.
Following the identification of genes of interest with PCR and sequence analysis, housekeeping genes; glyceraldehyde-3-phosphate dehydrogenase (gaP) and cell division protein gene ftsZ were determined to be stable together and were therefore selected for *E. coli* Figure 8.1 (a) (appendix), For *S. aureus* strains recombinase, A gene (recA) and DNA replication and repair protein gene recF was both used for normalization Figure 8.1 (b) (appendix). For *P. aeruginosa* recombinase A (recA) and adenylate kinase (adK) were used for normalization Figure 8.1 (c) (appendix). These housekeeping genes were selected because they are genes that are required for normal cellular functions and their expression levels are usually constant in both normal and abnormal conditions. The selected housekeeping genes were therefore used to normalise Cq values of treated and untreated samples.

**Gene and primer efficiency**

To determine gene and primer efficiency, genomic DNA templates for standard curves were quantified and diluted serially in 5 dilutions of 1:10. Triplicate of each template dilutions were used to give greater confidence in data. Resulting data was plotted to produce standard curves for each primer set. The amplification efficiency was \[10^{(-\frac{1}{\text{slope}})} - 1\]. A 0.99 or greater correlation coefficient (R2) was preferred. Acceptable range of efficiency is between 90 and 110 %. Table 8.5 (appendix) show data of gene and primer efficiency used in this study.

**Figure 4.4.** shows example of melt curve measurements of; *ompF*, Figure 4.4 (a-c) efficiency was 101 %, 0.99 R2, no primer dimers found and *norA*, Figure 4.4 (d-f)
efficiency of 96 %, 0.99 R2, no primer dimers found. The examples of process involved in the measurement of differential expression of genes are shown in Figure 8.3 and 8.4 (appendix).

![Figure 4.4. Example of gene efficiency measurement.](image)

The efficiency of each primer was measured using genomic DNA. a- c, *ompF* efficiency was 101 %, 0.99 R2 and no primer dimers and d-f *norA* efficiency of 96 %, 0.99 R2 and no primer dimers. Acceptable range of efficiency is between 90 and 110 % and R2 0.99 or greater.
The effect of sub-inhibitory concentrations of hydrogen peroxide on the expression of *E. coli* oxidative stress response and regulatory genes

To understand the mechanisms and genes involved in antibiotic cross-resistance previously observed in EcH2O2 in section 2.3.3, after being primed with hydrogen peroxide, gene specific primers were designed to compare gene expression profiles of suspected stress response genes in this strain with wild type EcATCC. The Cqs of each gene and endogenous controls (*gap* and *fitZ*) were calculated with qPCR, the Cqs were then normalized with the endogenous genes.

**Figure 4.5** shows gene expression of transporters and regulators in EcH2O2 using EcATCC as control baseline, strains were grown without antibiotic or 4 mg/l cephalothin (KF). Paired sample *t*-test comparing Cq values showed that there was a significant 30.5-fold downregulation of the *E. coli* porin genes *ompF* (*p*=0.01) in ECH2O2 when grown without cephalothin and 37.9-fold downregulation when grown in 4 mg/l cephalothin (*p*=0.028) **Figure 4.5 (a)**. This is interesting since OmpF is usually the porin of choice for β-lactam antibiotics to enter *E. coli*, therefore a downregulation of that level would contribute to the increased cross-resistance in the β-lactams used in this study. It is also interesting that it was also downregulated in the absence of cephalothin, suggesting *ompF* was permanently downregulated in EcH2O2 and its expression levels was not
antibiotic-dependent but as a result of being exposed to sub-lethal concentrations of hydrogen peroxide.

There was almost an expectation of a significant upregulation of *acrAB* due to observed increased susceptibility of EcH2O2 to both cephalothin and cephotixin in the presence of the efflux pump inhibitor thioridazine (*Section 3.3*). Also, the change in nucleotide and amino acid sequence observed in this chapter.

The *micF* gene which is the negative regulator of the *ompF* porin gene in *E. coli* was not significantly upregulated in EcH2O2 with or without cephalothin, suggesting it may not be the cause of the significant downregulation of *ompF* observed here Figure 4.5 (b). All other genes tested were uniformly downregulated in EcH2O2 in the presence of 4mg/l cephalothin and without the antibiotic, although not to significant levels, when compared to EcATCC under the same conditions Figure 4.5 (b).

This is an interesting trend as it seems these genes are normally upregulated in this strain (as seen in parent strain EcATCC).
Figure 4.5. Differential expression of *E. coli* stress response genes

Eleven outer membrane protein (OmpF) inner membrane transporters (AcrA, AcrB) and regulators (SoxR, SoxS, MICF, MarA, MarC, MarR and OxyR) were studied in EcH2O2. EcH2O2 strain was grown in either 4 mg/l cephalothin or without cephalothin, using wild-type EcATCC grown in the conditions as control baseline. The Cqs of each gene and endogenous controls were calculated with qPCR, the Cqs were normalised with endogenous controls (gaP and fitZ). (a), there was a significant 30.5-fold ($p=0.028$) and 37.9-fold ($p=0.01$) downregulation of *ompF* porin when EcH2O2 was grown without cephalothin and in 4 mg/l cephalothin respectively. (b), All other EcH2O2 genes were uniformly downregulated in comparison with EcATCC under the same conditions. (n=3; error bars = mean ± standard error of the mean).
The effect of sub-inhibitory concentrations of chlorhexidine on the expression of *S. aureus* norA and ImrS efflux genes

Following the cross-resistance to oxacillin previously observed in SaCHG after exposure to chlorhexidine, quantitative Realtime PCR was used to measure expression of efflux pump genes *norA* and *lmrS* previously identified and sequenced in Figure 4.2 (a) and (b) in both SaATCC and SaCHG. SaATCC was used as control baseline, strains were grown without antibiotic, 0.0625 or 0.125 mg/l oxacillin. The Cqs of each gene and endogenous controls (*recA* and *recF*) were calculated with qPCR, the Cqs were then normalized with the endogenous control genes.

Paired sample *t*-test comparing Cq values show that there was a significant 7-fold upregulation in *norA* gene in the presence of 0.125 mg/l oxacillin (*p* = 0.039) but not in 0.0625 mg/l of the antibiotic (*p* = 0.412) Figure 4.6, suggesting that the NorA efflux pump may be contributing to the oxacillin resistance observed in section 2.3.3. There was no significant change in the expression levels of the *lmrS* gene in either 0.0625 (*p* =0.431) or 0.125 mg/l (*p* =0.111) oxacillin. The results suggest that NorA and not LmrS pump may be contributing to the oxacillin resistance previously observed. Experiment was performed in both biological and technical triplicates (n=3).
The differential gene expression of *norA* and *lmrS* in oxacillin resistant strain SaCHG using wild type strain SaATCC as control baseline. Strains were grown in 0, 0.0625 and 0.125 mg/l oxacillin. The Cqs of each gene and endogenous controls (*recA* and *recF*) were calculated with qPCR, the Cqs were normalized with the endogenous controls. Paired sample t-test shows a significant 7-fold upregulation in *norA* in the presence of 0.125 mg/l oxacillin ($p=0.039$) but not in 0.0625 mg/l. There was no significant expression of the *lmrS* gene in both SaATCC and SaCHG in either 0.0625 mg/l or 0.125 mg/l oxacillin. ($n=3$; error bars = mean ± standard error of the mean).
The effect of sub-inhibitory concentrations of glutaraldehyde on the expression of *P. aeruginosa* efflux gene mexJ and its regulator mexL genes

Following observed cross-resistance to ciprofloxacin and sulphatriad in PaGTA after exposure to low concentration of glutaraldehyde in chapter 2, it was necessary to understand the mechanisms and genes that may be involved. Realtime PCR was used to compare differential expression of mexJ efflux and its transcriptional regulator mexJ in PaGTA using PaATCC as control base line. The Cqs of each gene and endogenous controls (recA and adK) were calculated with qPCR, the Cqs were then normalized with the endogenous control genes. The results summarized in Figure 4.7 show that when grown without ciprofloxacin, paired sample t-test comparing Cq values indicates no significant change in the expression levels of mexJ efflux gene (1.3-fold) (p=0.145) and its repressor mexL 1.5-fold (p=0.288) in the absence of antibiotics. Both mexJ and mexL genes showed no significant change in their expression in PaGTA when in the presence of 0.125 mg/l ciprofloxacin, with mexJ was more down regulated 0.5-fold than mexL 0.9-fold. n=3
The **mexJ** efflux pump gene and its regulator **mexL** were expressed in ciprofloxacin resistant strain PaGTA using wild type strain PaATCC as control baseline. Strains were grown without ciprofloxacin or without ciprofloxacin. The Cqs of each gene and endogenous controls (**recA** and **adK**) were calculated with qPCR, the Cqs were normalized with the endogenous controls. Paired sample t-test comparing Cq values showed no significant change in the expression levels of **mexJ** efflux gene (1.3-fold) \((p=0.145)\) and its repressor **mexL** 1.5-fold \((p=0.288)\) in PaGTA in the absence of antibiotics. Both **mexJ** and **mexL** genes showed no significant change in their expression in PaGTA when in the presence of 0.125 mg/l ciprofloxacin, with **mexJ** was more down regulated 0.5-fold than **mexL** 0.9-fold. \((n=3; \text{error bars} = \text{mean} \pm \text{standard error of the mean})\).
4.4. Discussion

Following observed stable cross-resistance to antibiotics in EcH2O2, SaCHG to β-lactams and PaGTA to ciprofloxacin in section 2.3.3, the observed effects of efflux pump inhibitors thioridazine and chlorpromazine on their antibiotic susceptibility in chapter 3, it is now necessary to know if the cross-resistance observed could be linked to changes in nucleotide sequences and expression levels of suspected antibiotic-resistant genes. Selected antibiotic resistance genes were identified in both wild-type and antibiotic-resistant strains using PCR, genes were then sequenced to identify changes in their nucleotide sequence. Gene expression profiles for the sequenced genes were compared between antibiotic-resistant and wild type strains using quantitative Realtime PCR.

The effect of sub-inhibitory concentrations of hydrogen peroxide on the expression of E. coli oxidative stress response and regulatory genes

The effectiveness of antibiotic drugs depends on their ability to attain intracellular concentrations high enough to inhibit their targets (Masi et al. 2019). In order to reach their targets, antibiotic drugs such as β-lactams must diffuse through bacteria outer membrane (Nikaido et al. 1977; Nikaido 1979; Harder et al. 1981; Masi et al. 2019). Crossing the outer membrane of Gram-negative bacteria is particularly tricky for these antibiotics due to the barrier created by their complex double cell envelope (Stavenger and Winterhalter 2014; Zgurskaya et al. 2015; Silver 2016), made up of a phospholipid and lipopolysaccharide (Nikaido 2003). In order the overcome these barriers, antibiotics
and other amphiphilic molecules must enter bacteria cells via transmembrane porins, of which *E. coli* has 3 well studied trimeric porins; OmpF, OmpC and PhoE (Hiroshi et al. 1977; Nikaido; 1979, Nikaido; Masi et al. 2019) which along with other oxidative stress response and antibiotic resistance genes are regulated by the transcriptional activators; the soxRS and oxyR regulons (Blanchard et al. 2007; Bruce 1991; Blanchard 2012). The present chapter looked at the changes in the expression levels of selected antibiotic resistant genes to see if the increased resistance to β-lactam antibiotics observed in EcH2O2 in **Section 2.3.3** could be linked to gene expression.

Comparing the gene expression profiles of antibiotic resistant strain EcH2O2 and susceptible parent strain EcATCC, significant down regulation of *ompF* porin was observed in EcH2O2 (30.5-fold without antibiotics and 37.9-fold in the presence of 4 mg/l cephalothin (**Figure 4.5(a)**). This significant downregulation of *ompF* was accompanied by an overall down regulation of the other genes studied, however not to significant levels. Looking at the expression profiles of these genes in parent strain EcATCC, it is clear that are they are normally expressed at slightly elevated levels, which suggests that EcH2O2 had deliberately suppressed their expression, perhaps to balance out the negative side effect of such a significant change in *ompF* expression. Broad suppression of stress response genes after acquisition of resistance is not that uncommon, in fact, this phenomenon was previously observed in the work of Händel *et al.* (Händel et al. 2013). When Händel *et al.* compared the gene expression profiles of amoxicillin resistant strain *E. coli* to the susceptible *E. coli* strain it was derived from,
they found a broad downregulation of such genes required for cellular stress response, the SOS response and genes required in DNA repair (Händel et al. 2013).

Exposure of bacteria to oxidative stress conditions such low concentrations of H₂O₂ can trigger the activation of protective mechanisms such as the SOS response, efflux pump activation and the downregulation of porins such as ompF, all which requires the use of cellular energy (Kim et al. 1997; Courcelle and Hanawalt 2003; Dwyer et al. 2009).

A significant downregulation of ompF gene possibly triggered a physiological imbalance which would lead to a metabolic burden, decreasing this imbalance by reducing the expression levels of other energy requiring genes would likely decrease the expected negative impact on the cell.

**The effect of sub-inhibitory concentration of hydrogen peroxide on ompF gene expression in EcH₂O₂**

It was previously demonstrated in chapter 2 that exposing *E. coli* to sub-inhibitory concentrations of H₂O₂ can cause increased resistance to cephalothin, cefoxitin and ampicillin, in this chapter quantitative Realtime PCR was used to show that ompF, one of the trimeric classical porin channels produced by *E. coli* (Masi et al. 2019) was significantly downregulated to 30.5-fold in *E. coli* cells that were primed with low concentrations of H₂O₂ and downregulated 37.9-fold more when these cells were grown in 4 mg/l cephalothin (**Figure 4.5 (a)**) and may contribute to the increased resistance observed. Reduced expression, modification or lack of porins which affects intracellular accumulation of antibiotics have been linked to reduced sensitivity to
antibiotics (Harder et al. 1981; Pages et al. 2008; Davin-Regli et al. 2008; James et al. 2009). Here we show that exposure of *E. coli* to increasing concentrations of H$_2$O$_2$ caused increased tolerance to β-lactams via significant reduced expression of *ompF* porin, which agrees with other studies where exposure of bacteria to increasing concentrations of antibiotic selected for strains that were not only resistant to cephalosporin and carbapenem due to reduced porin expression but were linked to the production of drug inactivating enzymes such as cephalosporinases and carbapenemases (Davin-Regli et al. 2008; Martínez-Martínez 2008; Blair et al. 2015; Nicolas-Chanoine et al. 2018). The reduced influx of β-lactams due to *ompF* down-regulation may allow only sub-inhibitory concentrations of antibiotic to enter the periplasmic space, a factor which have been shown to strongly induce β-lactamase production (Fisher and Mobashery 2014; Bush 2018) and a gateway for further development of resistant mechanisms to β-lactams (Nicolas-Chanoine et al. 2018).

Studies show that *ompF* expression can be repressed by *micF*, a small sRNA located upstream of the *ompC* promoter (Andersen et al. 1987), there was no significant increase in *micF* expression observed in this study, however The data presented show that as *micF* level increased in the presence of 4 mg/l cephalothin Figure 4.5 (b), suggesting the presence of antibiotics may affect its expression. Binding of MicF to the 5’ untranslated region of *ompF* prevents it’s post-transcriptional production (Andersen and Delihas 1990; Schmidt et al. 1995). The *micF* gene is regulated either through the high osmotic- *envZ ompR*2 component system (Aiba et al. 1987; Coyer et al. 1990; Ramani et al. 1994), *marA* due to antibiotic stress or SoxS due to oxidative stress (Chou
et al. 1993; Cohen et al. 1988; Chubiz and Rao 2011). It is also interesting to note that marA, (the promoter of the mar regulon) which was more expressed in this study although to significant levels, compared to micF (Figure 4.5 (b)) can also be activated by SoxS (Martin and Rosner 1997; Masi et al. 2019), suggesting that ompF could have been activated via SoxS or SoxS-MarA via oxidative response. From previous studies, it is understood that H$_2$O$_2$ activates both soxRS and the oxyR operons (Aslund et al. 1999; Manchado et al. 2000), both of which control the expression of over 100 oxidative stress response genes, one of which is micF (Demple1991; Blanchard et al. 2007; Blanchard et al. 2012). In the present study, a significant up-regulation of soxS gene was expected following ompF down-regulation as a result of oxidative stress, this was not observed. However, a 1.5-fold expression of sodA (Figure 4.5(b) ) an oxidative stress responder (Tang et al. 2002, Manchado et al. 2000) was observed in EcH2O2 when grown in 4 mg/l cephalothin , however previous studies suggest that the soxS gene performs its regulatory functions earlier on in the stress response period and returns to normal after that period is over (Viveiros et al. 2007).

The effect of sub-inhibitory concentration of hydrogen peroxide on acrA gene expression in EcH2O2

The exposure of E. coli to sub-inhibitory concentrations of H$_2$O$_2$ caused increased resistance to β-lactam antibiotics in section 2.3.3. The current study observed the significant down-regulation of ompF, ompF down-regulation in clinical isolates Enterobacteriaceae is usually accompanied by the increased expression in efflux activity
(Lavigne et al. 2013; Philippe et al. 2015; Masi et al. 2019), this was however not observed in this study. Here we observed a cytosine to thymine change in codon 255 (Figure 4.1 (d), corresponding with proline to serine change in position 255 (Figure 4.1 (e) and a 1.4-fold expression in acrA expression (Figure 4.5 (b)). The AcrA protein is the periplasmic component of the E. coli acrAB-tolC efflux pump (Zgurskaya and Nikaido 2000). Compounds such as aspirin and vanillin have been shown to induce increased levels of AcrA in cells, leading to antibiotic resistance (Nakamura 1968; Brochado et al. 2018). The acrA gene belongs to a group of genes that promote stress induced mutations (Al Mamun et al. 2012) and is known to control the opening and closing of the TolC expulsion channel by promoting interactions between AcrB and TolC (Gerken and Misra 2004; Wang et al. 2017). A reduced MIC of from 32 mg/l to 8 mg/l in cephalothin was previously observed in EcH2O2 when treated with the efflux pump inhibitor TZ in section 3.3.4, an action which may be caused by TZ binding to AcrA, as previously described by Abdali et. al (2017). The results obtained in chapter 3 suggested efflux mechanism to be a contributing factor to the reduced susceptibility to antibiotics observed. While a 1.4-fold expression of acrA is not significant enough on its own to cause increased tolerance, a synergistic relationship between a significantly reduced ompF and slightly upregulated acrA may be enough to pump out the small amount of antibiotics entering the cell. It is important to note that in this study, EcH2O2 was grown in 4 mg/l cephalothin as this was the concentration high enough to also support the growth of EcATCC which was used as control base line. Therefore a 1.4-fold expression of acrA at 4 mg/l cephalothin suggests that its expression in the presence of antibiotics could be concentration
dependent, since EcH2O2 is able to withstand increasing concentration of cephalothin as seen in subsequent growth curve experiment in section 5.3.6. This observation supports previous studies showing that expression of efflux pumps is tightly regulated (Grkovic et al. 2002), given the high energy cost (Lin. et al. 2018) and the side effect of a metabolic imbalance that may lead to fitness cost when they are overexpressed (Sánchez et al. 2002; Alonso et al. 2004; Piddock 2006).

The results seen here suggests that both efflux pump and porins are activated by exposure to low concentrations of H₂O₂ and may be responsible for the increased tolerance to β-lactam antibiotics used in this study.

The effect of sub-inhibitory concentrations of chlorhexidine on the expression of S. aureus norA and lmrS efflux genes

Staphylococcus aureus, a Gram-positive bacterium known for a range of infections; from minor skin infections to much serious life threatening ones like septicaemia, endocarditis and pneumonia (Lowy 1998; McDonnell and Russell 1999; Huet et al. 2008; Crossley et al. 2009). The spread of infections caused by S. aureus can be controlled by the use of varying biocides as decontaminants and disinfectants in hospitals, homes and laboratory environments (McDonnell and Russell 1999; McBain et al. 2002; Vali et al. 2008). The presence of a protective niche beyond the reach of lethal dose or continues exposure to sub-lethal concentrations may in time minimise the effects of the biocides and reduce their susceptibility (Boyce 2007; Smith et al. 2007; Huet et al. 2008). Chlorhexidine is one of the most used biocides in hospitals (Koljalg et al. 2002; Wand et
and also used in formulated products such as soaps and mouth wash (McDonnell and Russell 1999).

The effect of chlorhexidine against different strains of bacteria may vary, (Pacheco-Fowler et al. 2004; Thomas et al. 2005), but low level exposure and increase use have been linked to increase tolerance as well as cross-resistance to antibiotics (Vali et al. 2008; Huet et al. 2008; Maillard and Denyer 2009; Oggioni et al. 2013; Wesgate et al. 2016). Increased MIC to oxacillin, from 0.5 mg/l to 2 mg/l in SaCHG was previously observed in chapter 2 after continuous exposure to sub-inhibitory concentrations of chlorhexidine contained in a formulated product. Since most biocide related tolerance in *S. aureus* are linked to active efflux (Huet et al. 2008; Vali et al. 2008; Furi et al. 2013), it was important to know if efflux mechanism was activated in SaCHG. Oxacillin resistant strain SaCHG was previously treated with the efflux pump inhibitors TZ and CPZ in chapter 3, the effect was a reversal in the MIC of oxacillin from 2 mg/l to 0.25 with TZ and 0.5 mg/l with CPZ, suggesting efflux as a contributory mechanism to the cross-resistance to the antibiotic.

The *S. aureus* chromosome carries several multidrug efflux genes such as *norA*, *norB*, *norC*, *mrsA*, the tet38 and the *lmrS* (Lina et al. 1999; Truong-Bolduc et al. 2006; Ding et al. 2008; Fournier et al. 2000). Of all the genes listed, *norA* efflux gene is one of the most studied and its increased expression has been shown to not only induce resistance to antibiotics such as quinolones (Poole 2005; Patel et al. 2010) but to several other unrelated compounds (Yoshida et al. 1990; Ng et al. 1994; Truong-Bolduc and Hooper 2007).
To identify the efflux mechanism that may be contributing to the increased resistance to oxacillin, we compared **norA** and **lmrS** gene expression between SaATCC and SaCHG. Our results showed a significant 7-fold up-regulation ($p<0.05$) of **norA** gene (Figure 4.6) and 1-fold of **lmrS** gene in SaCHG (Figure 4.6), when bacteria were grown in 0.125 mg/l oxacillin compared to SaATCC, suggesting that **norA** and not **lmrS** gene was responsible for the observed reduced susceptibility to oxacillin. When bacteria were grown in 0.0625 and 0 mg/l oxacillin, there was no increased expression observed in either gene, indicating that expression of efflux pump in this instance was antibiotic concentration dependent and expression are upregulated only when needed. The data obtained in this study supports the tight regulation of Efflux expression as previously discussed in this chapter.

The expression of **norA** is known to be negatively regulated by MgrA, (Truong-Bolduc and Hooper 2007; Kumar et al. 2013) a multifunctional protein that also regulates the expression of **norB** and **norC** by binding to their promoters (Kumar et al. 2013). Increased expression of **norA**, especially after exposure to low concentrations of antimicrobial agents (Couto et al. 2008; Kumar et al. 2013) has been shown to be the result of mutation in the **norA** promoter, a mutation which reduces the binding of the negative regulator, allowing for uncontrolled increase in the expression of **norA** (Kaatz et al. 2005; DeMarco et al. 2007; Couto et al. 2008; Huet et al. 2008).

Sequencing of the **norA** gene, including the **norA** promoter region in SaCHG and SaATCC, showed no nucleotide change, deletion or substitution in the promoter region (Figure 8.2 appendix), there was however a single nucleotide, (adenine to thymine change) in
codon 313 (ATT to TTT) \textit{(Figure 4.2 (b))} in the norA coding region, resulting in a corresponding Isoleucine to Phenylalanine change is position 313 \textit{(Figure 4.2 (c))} in SaCHG. These findings suggest that mutations in other regions of norA other than the promoter region, may contribute to increase expression of the gene.

The effect of sub-inhibitory concentrations of glutaraldehyde on the expression of \textit{P. aeruginosa} efflux gene mexJ and its regulator mexL genes

The Gram negative bacteria \textit{P. aeruginosa} is innately resistant to most antibiotics and biocides amongst other compounds (Chuanchuen \textit{et al.} 2002; Olivares \textit{et al.} 2012; Jorgensen \textit{et al.} 2013; Lin \textit{et al.} 2018). A combined increase efflux and reduced influx has been blamed for the innate resistance in \textit{P. aeruginosa} (Hancock 1997; Zgurskaya and Nikaido 2000; Poole 2001). Biocides such as glutaraldehyde although its use is no longer common in most countries as previously mentioned in chapter 1 (Russell 1994; Cholley \textit{et al.} 2020), however it was previously used in both mechanical and automated endoscope disinfection (Russell 1994; Tschudin-Sutter \textit{et al.} 2011; Beilenhoff \textit{et al.} 2018; Cholley \textit{et al.} 2020). Resistance in \textit{P. aeruginosa} and other bacterial strains to GTA has previously been reported, (Kirschke \textit{et al.} 2003; Simoes \textit{et al.} 2006; Tschudin-Sutter \textit{et al.} 2011; Simões \textit{et al.} 2011; Kampf \textit{et al.} 2013) and an increase MIC to ciprofloxacin and sulphatriad after \textit{P. aeruginosa} was exposed to sub-inhibitory concentrations of GTA was previously observed in chapter 2. Where a 256 mg/l to 1024 mg/l increase in MIC in
sulphatriad and a 0.125 mg/l to 1mg/l in ciprofloxacin in PaGTA compared with ATCC 15442 was observed. Previous biocide related resistance in \textit{P. aeruginosa} have been attributed to active efflux (Chuanchuen \textit{et al.} 2002; Olivares \textit{et al.} 2012; Vikram \textit{et al.} 2015) and efflux pump inhibitors such as thioridazine have been shown to block efflux pumps and potentiate antimicrobial activities (Nikaido 1994; Mahamoud \textit{et al.} 2007; Pagès and Amaral 2009; Marino \textit{et al.} 2013; Rao \textit{et al.} 2018). In chapter 3, PaGTA was treated with known efflux pump inhibitor thioridazine, there was no observed change in the MIC of sulphatriad and ciprofloxacin, suggesting that efflux may not be responsible for the reduced susceptibility to antibiotics observed. To further confirm the results, the expression of \textit{mexJ} efflux gene and its regulator \textit{mexL} in both PaATCC and PaGTA in the presence of ciprofloxacin was compared, there was no increase expression in either gene.

The results obtained in this study suggests that active Efflux may not be responsible for the reduced antibiotic susceptibility observed here. Exposing \textit{P. aeruginosa} to sub-inhibitory concentrations of GTA may have triggered a cross-resistance to two antibiotics however, the mechanisms through which this has occurred has not been identified in this present study.

Further work such as looking at mutation in drug target sites, for example the \textit{gyrA/B} subunits or enzymatic degradation in relation to ciprofloxacin resistance may be carried out.
The data obtained in this chapter suggests that exposure to sub-inhibitory concentrations of biocide can trigger the upregulation of protective response genes to either prevent uptake of antibiotics or actively remove antibiotics from bacterial cells. While these mechanisms of resistance may be advantageous to resistant bacteria when antibiotic is present, studies show that some of these advantageous resistant mechanisms do come with some disadvantageous costs such as reduced growth and survival rate in the absence of antibiotics.

In the next chapter, comparative assays such growth curve analysis and biofilm growth will be used to compare parent and antibiotic resistant strains, this is to see if the observed increased antibiotic resistance and changes in gene expression affects bacterial fitness.
5. Chapter Five: Fitness cost of antibiotic resistance

5.1. Introduction

The term “use it or lose it” (Hall 2004) in reference to advantageous selective pressure favouring antibiotic resistant strains of bacteria, suggests that if the effector agents such as antimicrobials are removed, there should be a decline of the resistant strain in the population (Levin et al. 2000; Johnsen et al. 2009).

An example of this is shown in the versatility and adaptive mechanisms for colonising surfaces, which have contributed to the survival of microorganisms for millions of years (Kraigsley and Finkel 2009). The attachment and proliferation of bacteria on surfaces was first described by ZoBell and Allen in 1935 (Zobell and Allen 1935), who observed that bacteria would most likely colonise solid surfaces rather than remain in planktonic state (Zobell 1943). Bacteria uses biofilm to attach, proliferate and colonise surfaces (Hall-Stoodley et al. 2004; Tuson and Weibel 2013; Pavithra and Doble 2008). Even after colonization of surfaces, bacteria must be able to survive for long period of time, therefore being able to resist dryness is an important factor in their dissemination (Neely 2000; Smith et al. 1996). Bacteria such as P. aeruginosa require both flagella and twitching motility for the initiation and maturation of a biofilm which is important in their colonisation (O'Toole and Kolter 1998; Overhage et al. 2005), especially in infections such as cystic fibrosis, and device and ventilator related infections (Govan and Deretic 1996; Singh et al. 2000; Ramsey and Wozniak 2005; Pope et al. 2008). The
inability to form biofilm may impact their chances of survival, host colonisation and ability to cause disease.

Studies show mutations that confer resistance in bacteria may come with large fitness costs (growth disadvantage due to increased antibiotic resistance), where their growth and replication rates are reduced in environment without the selective agent, allowing for susceptible strains without fitness costs to outcompete them in the population (Gillespie and McHugh 1997; Andersson and Levin 1999). In a 2010 work by Deptuła and Gospodarek, a significant growth reduction in growth rate was observed in multidrug resistant *P. aeruginosa* when grown at 37°C in 2% proteose peptone for 20 hours compared to wild type strain (Deptuła and Gospodarek 2010). In another work, Wang *et al.* found that rifampicin resistance in *S. aureus* resulting from *rpoB* mutation caused reduced growth rate of the mutant strain compared to wild type (Wang *et al.* 2019).

Some antibiotic resistant mutations have been shown to have adverse effects on important biological cell functions, for example loss of function mutation in *P. aeruginosa* *nfxB* which caused an upregulation of *mexCD-oprJ* and fluoroquinolone resistance was associated with impaired motility in the resistant strain (Stickland *et al.* 2010). Other examples of mutations leading to antibiotic resistance that can affect fitness are described by Springer *et al.*, where resistance to aminoglycosides such as how the *rrs*-streptomycin resistance in *Mycobacterium smegmatis* affects ribosomal function by preventing the interaction between nucleotides 524G and 507C, an interaction which was described as essential for ribosomal function (Springer *et al.* 2001).
Though the use and expression of efflux pumps to expel toxic compounds from their cells is important in the ability of bacteria to exhibit antimicrobial resistance, overexpression of these can become disadvantageous, as both nutrient and metabolic intermediates may be expelled along with the toxic compounds (Webber and Piddock 2003). Overexpression may lead to metabolic imbalance resulting in a fitness cost (Sánchez et al. 2002; Alonso et al. 2004; Piddock 2006), for example in the work of Sánchez et al. several competitive assays were used to compare the fitness of multidrug mutants *P. aeruginosa MexABOprM-nalB* (K1111), *P. aeruginosa MexCDOprJ-nfxB* (K1112) to wild type strain *P. aeruginosa* ML5087. They found that compared to the wild type strain ML5087, *nalB* and *nfxB* mutant’s ability to survive in water (for *nfxB* significant at day 5 and 13) and dry surfaces was significantly impaired. However, biofilm formation was not reduced in the mutant strains, instead biofilm growth was significantly higher than that the wild type strain (Sánchez et al. 2002). The group also found that both *nalB* and *nfxB* mutants lost their ability to kill nematodes compared to the wild type strain which killed 80 % of nematodes when they were all grown at 20°C (Sánchez et al. 2002). Determining the fitness cost of any bacterial strain can be tedious and requires different experimental procedures and models, depending on the growth requirement, natural environment, host colonisation and mode of transmission (Pope et al. 2010). For example, if the fitness cost of a bacteria such as *P. aeruginosa* or *Burkholderia cepacia* is to be studied, it will be necessary to add a suitable biofilm assay (Pope 2008), as biofilm formation is part of the colonisation strategy of the bacteria, especially in infectious diseases such as cystic fibrosis (Singh et al. 2000). The gain of an
advantageous resistance mechanism, for example ability to survive in high antibiotic concentrations, that comes with a loss of the ability to form biofilm in *P. aeruginosa* will therefore be disadvantageous, because biofilm formation is required for it to survive within its host and cause disease (Singh *et al.* 2000; Pope *et al.* 2008; Pope *et al.* 2010). Fitness cost can also be tested by competitively measuring relative growth, reproductive and death rate between wild-type and antibiotic resistant bacterial strains (Bjorkman *et al.* 1998; Björkman *et al.* 1999).

This study has so far generated stable antibiotic resistant bacteria by exposing them to low concentrations of biocides, it will be interesting to see if this reduced susceptibility to antibiotics comes with fitness costs to the organisms when compared with wild type strains.

5.1.1. Chapter objectives

This chapter aims to see if there are fitness costs associated with the antibiotic resistance observed in the strains used in this study, after their exposure to low concentrations of biocides. This will be achieved by;

Comparing growth rate between wild-type and antibiotic resistant strains of bacteria, using 24-hour growth curves when bacteria are grown without antibiotics and in the presence of various concentrations of antibiotics,

Comparing the formation of biofilms between parent and primed strains using both crystal violet and glucose growth assays,
Measuring survival rates by testing survival in water between parent and primed bacterial strains.
5.2. Methods

5.2.1. Bacterial strains, storage and growth conditions

*Escherichia coli* ATCC 8739, EcH2O2, *Pseudomonas aeruginosa* ATCC 15442, PaGTA, *Staphylococcus aureus* ATCC 6538 strains and SaCHG were stored and grown as previously described in section 2.2.1

5.2.2. Reagents and chemicals

Crystal violet 0.1 % was obtained from BDH, ethanol 95 % was obtained from VWR and glucose powder was obtained from Fisher.

5.2.3. Preparation of inoculum

Bacterial strains were grown, and inoculum prepared and standardised as previously described in section 2.2.6

5.2.4. Comparison of growth of parent and tolerant strains

The standardised inoculum of parent and primed strains of bacteria (50 µl) was dispensed into each well of a 96-well plate already containing 50 µl of sterile MHB broth to give a final inoculum concentration of $5 \times 10^5$ CFU/ml. Standardised inoculum (100 µl) and sterile broth (100 µl) were dispensed into two wells of the 96-well plate as positive and negative control respectively. The inoculated 96-well plate was covered with optical adhesive film (ThermoFisher) to prevent evaporation and incubated in a SPECTRAmax plus spectrophotometer at 37°C for 24 hours. Absorbance at 600 nm was taken every
60 minutes, shaking for 5 seconds before each reading. Resulting data were plotted and generation times during the presumed log phase of growth were calculated from the graphs using the following formula:

\[ G = t \log \frac{b}{B} \]

Where \( B \) = number of CFU at the beginning of a time interval, \( b \) = CFU at the end of time interval and \( t \) = time intervals in minutes (Todar 2006).

Each experiment for each strain was conducted in technical triplicates and repeated on 3 separate days using different colonies.

5.2.5. Biofilm growth with crystal violet

Adapting the method of Reisner et al., overnight bacteria broth culture grown in a shaker incubator at 37 °C of both parent and primed strains was used to inoculate 100 µl of tryptone soya broth in 4 wells (per strain) of 96-well plate. The inoculated plates were incubated in a shaker incubator at 37°C at 200 RPM. After 24 hours of incubation, the culture was used to inoculate corresponding wells of a new 96-well plate containing 150 µl tryptone soya broth using sterile pipette tips attached to a multichannel pipette. Inoculated plates were placed in thick transparent plastic bags (to prevent broth from evaporating) and incubated at 37°C for 48 hours without shaking (Reisner et al. 2006). After 48 hours, wells were emptied by manually discarding the liquid into waste container. Pre-warmed (room temperature) 0.9 % NaCl (200 µl) was used to wash each well twice and then 200 µl of 0.1 % crystal violet solution was added to the wells and
left to incubate at room temperature for 15 minutes after which the crystal violet was
discarded, the wells were washed two times with 0.9 % NaCl solution. Ethanol (96 %,
170 µl) was added to each well to dissolve the crystal violet by pipetting up and down
with a multi-channel pipette.

Absorbance was measured at 590 nm in a plate reader. Each experiment was repeated
on three separate days, including positive (unstained bacterial growth wells) and
negative controls (broth alone).

5.2.6. Biofilm formation in 2 % glucose

Applying the method used by Chung et.al, (Chung et al. 2018), a single colony from a TSA
plate grown at 37°C for 18 hours, was used to inoculate glass test tubes containing 6 ml
TSB with 2 % (w/v) glucose. The inoculated tubes were incubated at 37°C with shaking
for 18 hours. Each test was performed in triplicate using different colonies and repeated
on three separate days using different colonies with negative controls (TSB).

5.2.7. Survival in water

Applying the method used by Pope et al.(Pope et al. 2010), overnight 1 ml MSB cultures
of parent and primed bacterial strains were centrifuged at 13,000 xg for 3 minutes. The
supernatants were removed and resulting pellets were washed three times in 1 ml
sterile 1X phosphate buffered solution (PBS). Approximately 1X10^8 CFU/ml were added
to 19 ml of sterile tap water. Aliquots (1 ml) were aseptically removed and exact CFU/ml
were determined by spiral plating method (Interscience for Microbiology). Aliquots
were taken on day 0, 1, 2, 5, 7, 8 and 9. Each experiment was carried out in biological and technical triplicates (n=3).

5.2.8. Spiral plate method

A 100 µl aliquot of each serial diluted sample was spread on triplicate TSA plates using an Interscience serial plater, tubes were cleaned before and between dilutions with 70% ethanol. Plates were incubated at 37 °C for 24 hours, after which colonies from 2 corresponding numbered grids were counted and used to ascertain CFU/ml.
5.2.9. Growth advantage in antibiotics

To further investigate the isolates with increased MIC to antibiotics after exposure to low concentrations of biocides observed in section 2.3.3, wild-type and antibiotic resistant bacteria were grown in varying concentrations of antibiotics to measure their growth.

The standardised inoculum of parent and primed strains of bacteria (50 µl) was dispensed into each well of a 96-well plate already containing 50 µl of the appropriate concentration of antibiotics (prepared as previously described in section 2.2.3) to give a final inoculum concentration of $5 \times 10^5$ CFU/ml. Standardised inoculum (100 µl) and sterile broth (100 µl) were dispensed into two wells of the 96-well plate as positive and negative control respectively. The inoculated 96-well plate was incubated and analysed as described in section 5.2.4.

5.2.10. Statistics

All tests were performed in technical and biological triplicates (unless when stated) and data are presented as means ± standard deviations, IBMS SPSS version 25 (Wagner III 2019) paired sample t-test was used to check significant difference between strains.
5.3. Results

Antibiotic cross-resistance was previously observed in EcH2O2 (β-lactams), SaCHG (oxacillin) and PaGTA (ciprofloxacin) after wild-type bacteria were exposed to sub-inhibitory concentrations of biocides (chapter 2). Gene expression profiles of selected antibiotic resistant genes (ompF porin gene in EcH2O2 and norA efflux gene in SaCHG) using quantitative Realtime PCR, showed significant expression fold change in these genes (Chapter 4). Data from previous studies indicates that advantageous mutations that confer antibiotic resistance to bacteria, may come with large fitness costs that may negatively affect their growth and replication in the absence of the selective agent. These associated fitness cost may allow susceptible strains without fitness costs to outcompete them in the population (Gillespie and McHugh 1997; Andersson and Levin 1999).

To understand if the observed antibiotic resistance and significant change in gene expression affects the fitness of these resistant strains, several competitive assays as growth curve, biofilm formation and survival were carried out in comparison to wild type strains the results obtained are discussed in this section.

5.3.1. Comparison of growth between parent and adapted strains

To compare the growth rate between antibiotic resistant and wild-type bacteria, both strains were grown without antibiotics in a SPECTRAMax plus spectrophotometer at
37°C for 24 hours. Absorbance at 600 nm was taken every 60 minutes. Resulting data were plotted and generation times were calculated from the graphs.

Results of 24-hour growth curve between parent and primed strains are shown in Figure 5.1 (panel a-c). Paired sample t-test was used to statistically compare any difference in growth between wild-type and antibiotic strains.

There was no significant difference (p>0.05) in the growth of EcATCC and EcH2O2 Figure 5.1, panel (a). Both EcATCC and EcH2O2 started exponential phase 2 hours after incubation and maintained this similarity throughout their stationary phase. The generation time of EcH2O2 was 57 minutes compared to EcATCC 56 minutes.

SaCHG grew significantly more quickly than SaATCC (p<0.05) Figure 5.1, panel (b). The calculated generation time for SaATCC was 46 minutes compared to 42 minutes for SaATCC. Please note the third biological samples for both SaATCC and SaCHG were omitted due to the plate reader not getting up to 37°C only during that run.

PaGTA grew significantly more than PaATCC (p<0.05) Figure 5.1 panel (c). PaGTA showed no visible lag phase (readings were taken at 60 minutes intervals), compared to 4 hours of PaATCC. The calculated generation time for PaGTA was 55 minutes compared to 53 minutes for PaATCC.
Figure 5.1. Comparison of growth between parent and antibiotic resistant strains

Wild-type and antibiotic resistant bacteria were grown at 37°C without antibiotics and absorbance readings was taken at 60 minutes intervals for 24 hours. (a). EcATCC and EcH2O2, there was no significant difference in their growth rate and generation time (56 and 57 minutes) (b). SaATCC and SaCHG, there was a significant difference in their growth rate ($p<0.05$), generation time was 46 and 42 minutes respectively, and (c) PaATCC and PaGTA, PaGTA grew significantly more than PaGTA ($p<0.05$) however generation time was 55 and 53 minutes respectively (n=3; mean ± standard error of the mean) except for (b) (n= 2; mean ± standard error of the mean).
5.3.2. Biofilm growth

Bacteria use biofilm to attach, proliferate and colonise surfaces (Hall-Stoodley et al. 2004; Tuson and Weibel 2013; Pavithra and Doble 2008), therefore if their ability to form biofilm is impaired, it can negatively impact their chances of survival, host colonisation and ability to cause disease (Pope et al. 2010). The strains used in this study have become resistant to antibiotics after exposure to low concentrations of biocides, since antibiotic resistance mutation sometimes comes with fitness costs such as reduced biofilm growth, it was therefore important to compare biofilm formation between wild type and antibiotic resistant strains.

To compare static biofilm formation between antibiotic resistant and wild-type bacteria, two methods were used; crystal violet assay, a quantitative method which relies on the absorbance of biofilm growth stained by crystal violet and a 2 % glucose assay, which relies on the qualitative observation of biofilm formation on the surface of TSB containing 2 % glucose. Using both methods allows for comparison and confirmation as well ensuring results are accurate.

5.3.3. Crystal violet assay of biofilm growth in all strains

For this method, overnight bacteria 150 µl broth culture grown at 37 °C of wild-type bacteria (EcATCC, SaATCC and PaATCC), antibiotic resistant bacteria (EcH2O2, SaCHG and PaGTA) and biocide tolerant bacteria (PaBKC) were grown at 37°C for 24 hours. After which they were washed, surface attached cells were stained with crystal violet, extracted with 96 % ethanol and absorbance was measured at 585 nm. Tests were
carried out in biological triplicates and technical quadruplets. IBMS SPSS version 25 (Wagner III 2019), paired sample t-test was used to investigate statistical differences in biofilm growth between wild-type and antibiotic resistant strains.

**Figure 5.2** compares biofilm growth in wild type, biocide tolerant and antibiotic resistant strains. Biofilm growth was observed in all strains tested, the average absorbance for parent strain EcATCC and antibiotic resistant strain EcH2O2 was 3.33 and 3.50 nm respectively, their absorbance was comparable to those of parent strain PaATCC, antibiotic resistant strain PaGTA and biocide tolerant strain PaBKC average absorbance of was 3.14, 3.49 and 3.43 respectively. While both SaATCC and SaCHG produced biofilm with average absorbance of 2.83 and 2.47 nm respectively and paired sample t-test comparing the two showed $p>0.05$, they were however the lowest biofilm producers when compared to the other 5 strains studied.

No significant difference in biofilm growth was observed between each parent and biocide adapted pair, suggesting that the antibiotic and biocide tolerance observed previously observed in **section 2.3.3** has not impaired their ability to form biofilm.
To compare biofilm growth between wild-type and biocide tolerant and antibiotic resistant strains, 48 hours biofilm produced were stained with 0.1 % crystal violet and absorbance was read at 590 nm. For EcATCC and EcH2O2, average absorbance was 3.33 and 3.50 nm respectively, there was no significant difference in biofilm growth, PaATCC and PaGTA average absorbance was 3.14 and 3.49 nm respectively, PaBKC average absorbance was 3.43 nm, and SaATCC and SaCHG average absorbance was 2.83 and 2.47 nm respectively. No significant difference was observed between the strains (n=3; error bars = mean ± standard error of the mean).

Figure 5.2. Comparison of biofilm formation in parent and biocide adapted strains
5.3.4. Biofilm growth in 2 % glucose

To confirm the results obtained by crystal violet assay, biofilm growth was observed in 2 % glucose a method previously used by Chung et al., (Chung et al. 2018). A single colony of each wild-type and antibiotic resistant bacteria was used to inoculate 6 mls of TSB containing 2 % glucose. After growth and 37°C, biofilm growth on the surface of the broth was observed and compared.

In the presence of 25 glucose (Figure 5.3), biofilm growth was observed on the surface of both EcATCC (a) and EcH2O2(b) compared to negative control (c). There was however no difference in the level of biofilm formed between the strains. Both SaATCC (d) and SaCHG (e) also showed biofilm formation with compared to control (f) no difference between the two strains. Of all the strains tested *P. aeruginosa* strains PaATCC (g) PaGTA (h) and PaBKC (i) formed considerably more biofilm than the rest, there was however no observable difference in biofilm formation parent strain PaGTA and the two-biocide adapted strains PaGTA and PaBKC.

The results obtained here confirms the crystal violet data, that the amount of biofilm growth was not visibly different between wild-type, biocide tolerant and antibiotic resistant strains.
To confirm and compare biofilm growth between wild-type and antibiotic resistant strains, one colony of each strain was grown at 37°C in 6 ml TSB containing 2% glucose. Biofilm growth on the surface of the broth was observed after 24 hours incubation. a-c. EcATCC, EcH2O2 and negative control (broth), there was no observable difference in biofilm growth, d-f. SaATCC, SaCHG and negative control (broth), there was no observable difference in biofilm growth and g-j. PaATCC, PaGTA, PaBKC and negative control (broth), there was more biofilm growth compared with other strains, but there was no observable difference in PaGTA and PaBKC compared to PaATCC. n= 3.
5.3.5. Survival in water

For nosocomial bacteria such as *E. coli*, *S. aureus* and *P. aeruginosa* water is an important habitat and route of dissemination (Vaz-Moreira *et al.* 2014). Therefore, any mutation that impairs their ability to survive in water may reduce their level of transmission (Sánchez *et al.* 2002). To investigate if the antibiotic resistance observed (Chapter 2) and the observed changes in the gene expression levels of antibiotic resistant genes (chapter 4) impairs the survival of antibiotic resistant bacteria compared to wild-type strains.

To compare the survival of wild-type (*EcATCC, PaATCC, SaATCC*) antibiotic resistant (*EcH2O2, PaGTA, SaCHG*) and biocide tolerant strain (*PaBKC*) in water, 1x10$^8$ CFU/ml from overnight culture grown at 37°C was washed by centrifugation in sterile 1x PBS. Washed cells were used to inoculated in sterile 10 ml tap water, for a week and samples were taken and plated for colony counts (spiral plater see section 5.2.8) at days 0, 1, 2,5,7,8 and 9 to compare their survival rate. Dilutions were plated in triplicates; experiments were repeated in biological triplicates (using different colonies) and CFU/ml was used to plot graphs.

**Figure 5.4** panel *(a-d)* shows the results of colony counts from all strains plotted on bar charts. EcATCC and EcH2O2, both strains survived up to nine days in tap water, there was no significant difference (*p*>0.05) in CFU/ml in both strains on all days tested *(a)*.

Of all the strains tested both SaATCC and SaCHG had the least survival rate as they both the not survive the full nine days. SaATCC however survived up to 24 hours longer (eight days) in water than SaCHG (seven days) *(b)*, there was however a significant increase (*p*<
0.05) in CFU/ml in SaCHG on day two compared to SaATCC on that day, no significant difference was observed on all other days tested.

Parent strain PaATCC and antibiotic resistant strain PaGTA survived nine days in water (c) and their growth was comparable biocide tolerant strain PaBKC which all survived up to nine days in water (d) PaBKC survived nine days in water with. There was no significant difference ($p > 0.05$) in CFU/ml between PaATCC, PaGTA and PaBKC on all the days tested.
To compare survival rate in water between wild-type and antibiotic resistant strains, sterile 19 mls tap water was inoculated with 1 ml of $1 \times 10^8$ washed bacterial cells. Samples were taken for seven days. Dilutions made were plated and incubated at 37°C, CFU/ml was calculated and plotted. (a) EcATCC and EcH2O2 both survived 9 days in tap water, no significant difference ($p>0.05$) in CFU/ml on all days tested, (b) SaATCC survived up to eight days in water compared to SaCHG 7 days, (c) parent strain PaATCC and antibiotic resistant strain PaGTA both survived 9 days in water and their growth was comparable to that of biocide tolerant strain PaBKC (d) which also survived up to nine days in tap water. (n=3; error bars = mean ± standard error of the mean).

**Figure 5.4. Survival in water assay**
5.3.6. Growth advantage in antibiotics

This experiment was performed to confirm the increased MIC to antibiotic observed in EcH2O2, SaATCC and PaGTA chapter 2 and to compare their potentially advantageous growth in increasing antibiotic concentrations compared to wild-type strains. Both wild-type and antibiotic resistant strains were grown in varying concentrations of cephalothin, oxacillin and ciprofloxacin at 37°C, optical density was measured every 60 seconds for 24 hours and data obtained was used to generate growth curves and calculate generation time.

**Escherichia coli strains**

Wild-type EcATCC and antibiotic resistant strain EcH2O2 were grown at 37°C in varying concentrations of cephalothin (1, 2, 4, 8, 16, 34 and 64 mg/l), results are shown in Figure 5.5. Both strains grew in 1 to 4 mg/l cephalothin, no further growth of EcATCC was observed in 8, 16, 32 and 64 mg/l cephalothin, confirming our previous observation that EcH2O2 was indeed resistant to cephalothin and could withstand higher concentrations of the antibiotic. Generation time for EcATCC in 1 to 4 mg/l cephalothin was; 54, 43 and 52 minutes. No visible lag phase was observed in EcH2O2 from 2 to 32 mg/l, however there was a prolonged lag phase of 8 hours at 64 mg/l. Generation time for EcH2O2 in 1 to 64 mg/l cephalothin was; 54, 58, 57, 58, 66, 55 and 53 minutes.
Wild-type EcATCC and antibiotic resistant strain EcH2O2 were grown in increasing concentrations of cephalothin, absorbance was taken at 60 minutes intervals. Both EcATCC and EcH2O2 grew in 1 to 4 mg/l cephalothin, no visible growth of EcATCC was observed in 8,16,32 and 64 mg/l cephalothin. There was a prolonged lag phase of 8 hours when EcH2O2 was grown in 64 mg/l cephalothin. (n=3; error bars = mean ± standard error of the mean).

**Figure 5.5. Growth advantage of resistant strain EcH2O2 in cephalothin**
Staphylococcus aureus strains

Wild-type SaATCC and oxacillin resistant strain SaCHG were grown at 37°C in 0.125, 0.25, 0.5 and 1 mg/l oxacillin to compare their growth in the presence of the antibiotic and to confirm the increased resistance to oxacillin previously observed in chapter 2. Results are shown in Figure 5.6. SaATCC only grew in 0.125 mg/l oxacillin with a prolonged lag phase lasting up to 13 hours, there was no visible growth observed in 0.25 to 1 mg/l. SaCHG grew in 0.125 to 1 mg/l oxacillin, generation time of SaCHG at varying concentrations of oxacillin was 67, 58, 41 and 55 minutes.
Figure 5.6. Growth advantage of antibiotic resistant strain SaCHG in oxacillin

Wild-type SaATCC and oxacillin resistant strain SaCHG were grown in 0.125, 0.25, 0.5 and 1 mg/l oxacillin. Absorbance was taken at 60 minutes interval and data was plotted. SaATCC only grew at 0.125 mg/l oxacillin with a prolonged lag phase of 13 hours. SaCHG grew in 0.125 to 1 mg/l oxacillin with generation time of 67, 58, 41 and 55 minutes. (n=3; error bars = mean ± standard error of the mean).
*Pseudomonas aeruginosa* strains

Wild-type PaATCC and ciprofloxacin resistant strain PaGTA were grown at 37°C in 0.125, 0.25, 0.5 and 1 mg/l ciprofloxacin to compare their growth in the presence of the antibiotic and to confirm the increased resistance previously observed in chapter 2. Results are shown in Figure 5.7, PaATCC grew in 0.125 and 0.25 mg/l ciprofloxacin with a prolonged lag phase, there was no visible growth observed in 0.5 and 1 mg/l. PaGTA grew in 0.125 to 1 mg/l ciprofloxacin. Generation time of PaATCC in 0.125 and 0.25 mg/l ciprofloxacin was 52 and 53 minutes and generation time for PaGTA in 0.125 to 1 mg/l ciprofloxacin was 59, 57, 59 and 54 minutes.
Figure 5.7. Growth advantage of resistant strain PaGTA in ciprofloxacin

Growth of wild-type PaATCC and ciprofloxacin resistant strain PaGTA at 37°C with absorbance taken at 60 minutes intervals. PaATCC grew in 0.125 and 0.25 mg/l (generation time: 52 and 53 minutes), there was no visible growth in 0.5 and 1 mg/l ciprofloxacin. PaGTA grew in 0.125 to 1 mg/l ciprofloxacin (generation time; 59, 57,59 and 54 minutes) (n=3; error bars = mean ± standard error of the mean).
5.4. Discussion

In order to test if the cross-resistance to antibiotics observed in resistant strains (EcH2O2, SaATCC and PaGTA) observed in chapter 2 and the significant down regulation of *ompF* in EcH2O2 and upregulation of *norA* in SaCHG comes with a fitness cost, competitive growth experiments comparing them with wild type strains (EcATCC, SaATCC and PaATCC) were carried out. Strains were tested for difference in growth rate, ability to form biofilm and days of survival in water.

### 5.4.1. Fitness cost of antibiotic resistance on:

**Growth**

Antibiotic resistance in bacteria can sometimes come with non-specific metabolic burdens, which can contribute to some biological fitness costs such as reduction in their growth rate (Deptuła and Gospodarek 2010), as was previously discussed in the introduction section of this chapter. This was highlighted in previous work carried out by Wang *et al.* where they showed that rifampicin resistance in *S. aureus* caused by *rpoB* mutation resulted in the reduced growth rate of mutant strain compared to parent strain (Wang *et al.* 2019). Reduced growth was however not observed in the present study, when growth rate was compared between antibiotic resistant strains (EcH2O2, SaCHG and PaGTA) and wild type strains (EcATCC, SaATCC and PaATCC). While there was no significant difference between EcATCC and EcH2O2, SaCHG and PaGTA either grew significantly more than their wild type counterparts or had comparable growth, indicating a growth advantage rather than the expected disadvantage in the absence of
antibiotics. The data presented in this study indicates their selective advantage of antibiotic resistance has not left our resistant strains deficient enough to be outcompeted by the wild type strains as previously suggested (Gillespie and McHugh 1997; Andersson and Levin 1999). While this has been documented to sometimes happen as was described in the work of Deptula and Gospodarek where they compared the expression of virulence factors such as growth rate, polystyrene adhesion and Elastase activity between 75 multidrug resistant and 75 drug sensitive clinically isolated *P. aeruginosa* strains. They found that the multidrug resistant strains showed reduced growth rate, produced less slime and elastase when compared to antibiotic susceptible strains (Deptula and Gospodarek 2010), the data collected in the present study suggests that this may not always be the case.

Biofilm formation

Bacterial biofilm is associated with 65% hospital-related infections (Kumar et al. 2019) and contribute to over 80% of human microbial infections (Kumar et al. 2019). In diseases such as cystic fibrosis and device and ventilator related infections, biofilm formation is important in bacterial colonization (Govan and Deretic 1996; Singh et al. 2000; Ramsey and Wozniak 2005; Pope et al. 2008). Therefore, any mutation that impairs biofilm formation will be a fitness burden to the bacteria. While the effects of antibiotic resistance on bacterial biofilm formation vary, for example in the study of Sun et al. they reported reduced biofilm production in only 54.5% of their β-lactam resistant *P. aeruginosa* strains tested, while there was increased biofilm growth in the remaining
36.4 % when compared with wild type strains (Sun et al. 2013). There are several studies which associate changes to biofilm growth with antibiotic resistant phenotype. An example is the study performed by Choi and Ko in 2015, where colistin resistant *Klebsiella pneumoniae* strains 07-B-060R and 13703-3487R showed reduced biofilm growth compared to the wild type strains (Choi and Ko 2015). This present study showed no significant difference when biofilm growth was compared between antibiotic resistant and wild type strains. The data presented in this study agrees with that of Kugelberg et al. where biofilm growth was not reduced in quinolone resistant *P. aeruginosa* (Kugelberg et al. 2005), which is comparable to the result of fluoroquinolone (ciprofloxacin) resistant strain PaGTA whose biofilm production was comparable to parent strain PaATCC.

**Survival in water**

Water is an important environmental habitat for bacterial, it is also a major route for bacterial dissemination (Vaz-Moreira et al. 2014). Nosocomial bacteria such as *P. aeruginosa* are present in water reservoirs (Römling et al. 1994), their ability to survive in water is therefore important in their transmission (Sánchez et al. 2002). Upregulation of multidrug efflux pump MexCD-OprJ in *nalB* mutant *P. aeruginosa* was previously associated with significantly impaired their survival in water when compared with wild type *P. aeruginosa* ML5087 (Sánchez et al. 2002), this was however not observed in most of the antibiotic resistant strains used in this study except for SaCHG. When grown in sterile tap water, growth of both wild type and antibiotic resistant strains were
comparable in all other strains tested except for SaCHG, the data presented in this study show that SaATCC survived for 24 hours longer than SaCHG. Perhaps the increased resistance to oxacillin (observed in section 2.3.3) resulting from upregulation of the norA efflux pump observed in section 4.3.3, after being exposed to low concentrations of chlorhexidine has negatively impacted SaCHG surviving longer in water.

5.4.2. Reduction of biological burden

It is interesting that no observable fitness costs were identified in all the antibiotic resistant strains tested compared to their wild type counterparts. Studies shows that while resistant bacteria may initially suffer a fitness cost, reduction of these costs may be favoured by natural selection which would either select for less costly mutants at the resistance loci (Levin et al. 1997) or select for mutant at a different locus that can modify the costs (Bouma and Lenski 1988). The selection of mutants at different locus which can modify initial fitness costs was demonstrated by Bouma et al. in their 1998 work, in their experiments they observed that an E. coli strain which initially incurred reduced fitness in the absence of antibiotic, after being transformed with a non-conjugate plasmid pACYC184 (encoding resistance to tetracycline and chloramphenicol) became fitter than it’s plasmid-free and plasmid carrying ancestors after 500 generations in the presence of 10 µg/ml chloramphenicol. The observed increased fitness after 500 generations was attributed to changes in the bacterial chromosome but not in the plasmid (Bouma and Lenski 1988).
In a study by Schrag and Perrot, it was demonstrated that fitness cost caused by chromosomal resistance can be reduced through natural selection without any adverse effect on the initial resistance. In their study, the initial fitness cost to streptomycin resistant \( E. \ coli \) due to two different \( rpsL \) mutations was reduced after 180 generations in the absence of antibiotics. The reduction of fitness cost in this instance was attributed to the presence of compensatory mutations in different genes (Schrag and Perrot 1996; Levin et al. 1997).

The absence of observable significant biological fitness costs may be because the mutations causing increased tolerance to antibiotics may have been compensated for by a secondary mutation (Andersson and Hughes 2010; Knopp and Andersson 2015). Most mutations responsible for antibiotic resistance in bacteria, are often deleterious in drug free environments (Schrag et al. 1997; Sousa et al. 2011) because they may target proteins involved in key cellular functions, examples are the ribosomal target in \( rpsL \)-streptomycin resistance in \( Salmonella \ typhimurium \) which resulted in reduced protein synthesis and bacterial growth (Maisnier-Patin et al. 2002) and RNA polymerase target in rifampicin resistant \( P. \ aeruginosa \) (Reynolds 2000).

It was previously shown in chapter 2 that continuous exposure of \( E. \ coli \) to sub-inhibitory concentrations of \( H_2O_2 \) may have selected for a significant down-regulation of the porin gene \( ompF \) (Section 4.3.3) that allows them to grow in the presence of antibiotics such as \( \beta \)-lactams, as was previously shown in literature (Ferenci 2005; Davin-Regli et al. 2008; Phan and Ferenci 2017). Gram-negative bacteria such as \( E. \ coli \) rely on the uptake of nutrients and essential metabolites via porins such as OmpF, therefore the significant
reduction in such porins can cause nutrient deficiency that may have significant fitness cost (Ferenci 2005; Davin-Regli et al. 2008; Phan and Ferenci 2017). However, studies show that compensatory mutations can cause the upregulation of other major porin (or minor porins) which will replace the down regulated porin and continue to carry out the function of nutrient uptake. This was shown in the work of Knoop et al. where the deletion of the two *E. coli* classical porins; *ompF* and *ompC* (Knopp and Andersson 2015) increased resistance to antibiotics and caused two compensatory mutations that activated the functions of PhoE porin and overexpression of the small porin *chiP* (chitoporin), in order to offset the initial reduced growth rate (fitness cost) observed (Knopp and Andersson 2015). This may explain how EcH2O2 is able to compete with and outgrow parent strain with or without antibiotics and also suggests that bacteria can choose between when survival is more important; by down-regulating certain functions like the exclusion of antibiotics or employing different porins when nutrient is required for growth by trading one off for the other as required (Phan and Ferenci 2017).

Overexpression or non-regulated expression of efflux pumps has high energy requirements and are likely to result in metabolic imbalance leading to fitness costs (Martinez et al. 2009; Stickland et al. 2010; Alvarez-Ortega et al. 2013; Olivares et al. 2014). So, it makes more sense to express efflux only when required (Olivares et al. 2014) as could be seen by the difference in *norA* expression when SaCHG was grown in 0, 0.0625 and 0.125 mg/l oxacillin (Section 4.3.3), only when faced with a threatening concentration of oxacillin did significant expression of *norA* occur. If *norA* was only significantly expressed in SaCHG at higher concentrations of oxacillin, how does it
remove other unwanted compounds from its cells when grown without oxacillin, without incurring a fitness cost? Studies shows that \textit{S. aureus} also expresses other related efflux pumps such as \textit{norB}, \textit{norC} and \textit{norD} which can be highly expressed in the absence of \textit{norA} (Ding \textit{et al.} 2008; Ding \textit{et al.} 2012), therefore allowing the bacteria to continue to survive in oxacillin-free environment while still able to remove toxic compounds (Ding \textit{et al.} 2008).

5.4.3. Clinical implications of low-cost resistance

Bacteria with antibiotic resistant mutations are often thought to be outcompeted by their more sensitive counterpart in antibiotic-free environment, due to fitness cost that may impair growth and replication rate (Levin \textit{et al.} 1997; Johnsen \textit{et al.} 2009; Melnyk \textit{et al.} 2015). If antibiotic resistant bacteria are disadvantaged in the absence of antibiotics, a strategic way of eliminating them will be stopping the use of said antibiotics (Melnyk \textit{et al.} 2015). A depletion in the occurrence of resistant strains have been associated with reduced use of antibiotics in epidemiological studies. In one example country-wide prohibition in the use of certain classes of antibiotics in Cuba, lead to reduced resistance to most of the prohibited antibiotics (Calva \textit{et al.} 1996). In another example, when Hungarian doctors reduced the prescription of penicillin for pneumococcal infections, there was a decline in occurrence of penicillin resistant \textit{Streptococcus pneumoniae} strains (Nowak 1994).

While reduced use of antibiotics correlates with the decline of some antibiotic resistant strains in some epidemiological studies, clinical studies show varied outcome (Enne \textit{et
al. 2001; Arason et al. 2002). In some studies, the antibiotic resistant strains persisted in the population, an example of such is the study done in Royal London Hospital between 1991 and 1999. In that study, the prescription of sulphonamide was reduced from 320,000 in 1991 to 7,000 in 1999, in a bid to combat the occurrence of sulphonamide resistant E. coli, there was however no decline of the resistant strain in the population (Enne et al. 2001).

The examples discussed here, and the data obtained from the present study suggests that removal of antibiotics after occurrence of resistance does not always reverse the resistance and the resistant strains don’t always get outcompeted. As discussed previously in this chapter, while initial fitness cost may affect an antibiotic resistant strain, that cost can be phased out or reduced by natural selection after several generations without reversing the resistant genotype (Schrag and Perrot 1996; Levin et al. 1997).

5.4.4. Growth advantage in antibiotics

In the presence of their tolerant antibiotic concentrations, all primed strains showed significantly greater growth compared to their parent counterparts, confirming the increased MIC in antibiotics previously observed in these strains in section 2.3.3 and demonstrating their advantages in antibiotic survival. Growth started almost immediately in primed strains in the presence of antibiotics, with reduced or no lag phases as the antibiotic concentrations increased, however an extended lag phase was observed in EcH2O2 when grown in 64 mg/ cephalothin (a concentration higher than it
was used to). Reduction in porins have been known to completely block the influx of β-lactams (Pagès et al. 2008; Masi et al. 2017; Masi et al. 2019), which could be seen in further *ompF* down-regulation in EcH2O2 when grown in 4 mg/l cephalothin compared to when grown without the antibiotic (37.9-fold and 30.5-fold downregulation respectively), this may explain why EcH2O2 continue to grow in higher concentrations of the antibiotic.

While there are no observable fitness cost in the growth assays carried out in this study, it will be good to bear in mind that these conditions were *in vitro* therefore, results from *in vivo* conditions were animal models are used such as nematode killing may differ (Sánchez et al. 2002; Pope et al. 2010).
6. Chapter six: General discussion and conclusions

6.1. Background

The prevalence and rise in multidrug resistant bacteria have rendered most conventional antimicrobials ineffective in infection control (Walsh and Toleman 2011; Roca Subirà et al. 2012; Tuon et al. 2012). Antimicrobial resistance is now the leading cause of death worldwide (Erickson et al. 2017) as a result, 10 million antimicrobial-resistance related death have been predicted to occur by the year 2050 (Review on Antimicrobial Resistance 2014; O’Neill 2014). Biocides have long been used to control the spread of infections (Russell 2002; Maillard 2002; Morente et al. 2013), they are also added to cosmetics as preservatives, used as pesticides, antiseptics and disinfectants (Maillard 2002; Gilbert and McBain 2003; Knapp et al. 2015).

Public awareness to infection control, for example the present day COVID-19 pandemic where the reported great increase in the purchase and use of disinfectant and hand sanitisers (Berardi et al. 2020; European Parliament 2020; Hegarty 2020) can contribute to the uncontrolled wide-spread use of biocide and biocidal products especially at below in-use concentrations. This is a cause for concern because the use of any antimicrobials at sub-inhibitory concentrations can create selective pressure that will favour the development of adaptive strains (McBain and Gilbert 2001; Fraise 2002; SCENIHR 2010; Maillard et al. 2013 Knapp et al. 2015). The connection between the use of sub-inhibitory concentrations of biocides and increased tolerance have previously been demonstrated in laboratory studies (Walsh et al. 2003; Escalada et al. 2005; Christensen
et al. 2011; Knapp et al. 2013; Bock et al. 2016). In some cases, increased tolerance of bacteria to biocides after low level exposure has caused cross-resistance to some antibiotics (Wand et al. 2016; Kurenbach et al. 2015).

Since increased tolerance to biocide can be linked with antibiotic resistance (Chuanchuen et al. 2001; Braoudaki and Hilton 2004; Tkachenko et al. 2007; Soumet et al. 2012; Slayden et al. 2000; Wand 2017), it is important to understand the mechanisms that are activated during the adaptive process (Fernández et al. 2011).

Most adaptive resistance mechanisms are only temporary survival strategies for bacteria and are therefore short-lived (Adam et al. 2008). However, it is the heterogeneity brought about by the adaptation process that is problematic because it drives evolution (Motta and Aldana 2015). While adaptive resistance is understood to be a non-genetic response and a temporary survival tactic employed by bacteria (Erickson et al. 2017), the divergence that arises from the adaptive process may involve both genetic (Tenaillon et al. 2012; Toprak et al. 2012) and non-genetic mechanisms (Fong and Palsson 2005; Adam et al. 2008; López-Maury et al. 2008).

6.2. The role of concentration gradient on the evolution of resistance

The generation of stable antibiotic resistant mutants; EcH2O2, SaCHG, PaGTA and stable biocide tolerant strain PaBKC using biocide concentration gradient (gradient plate method) section2.2.9, highlights the role of concentration gradient in the development of resistance. The idea that concentration gradient plays an important role in the
evolution of drug resistance is not novel (Kepler and Perelson 1998; Burkala et al. 2005) and this study further adds to that idea.

Antibiotic resistance often requires the accumulation of different mutations (Lipsitch 2001), the survival of a single bacterium in a homogenous environment would therefore be reliant on its ability and speed in acquiring more than one of the required mutations before it is eliminated (Baquero et al. 1998; Mayers 2009). When compared to an heterogenous environment which would allow the bacteria to survive in safe niches long enough to acquire the required survival mutations one at a time (Baquero et al. 1998; Mayers 2009; Hermsen et al. 2012).

The observed cross resistance with antibiotics in the present study agrees with previous concerns and findings and reiterates the connection between the use of sub-inhibitory concentrations of biocides and antibiotic resistance (Knapp et al. 2013; Bock et al. 2016; Fraise 2002; SCENIHR 2010). At sub-inhibitory concentrations of antimicrobials for extended periods, the chance of developing mutations that can cause reduced susceptibility may be higher compared to when exposed to lethal concentrations (Gullberg et al. 2011; Hughes and Andersson 2012; Andersson and Hughes 2014).

6.3. Activated mechanisms of cross-resistance

In order to test the mechanisms that could have been activated during biocide adaptation, efflux activity and the effect of β-lactamase were tested in EcH2O2, SaCHG and PaGTA in section 3.3. The results presented in section 4.3.3 of a 7-fold increase upregulation of the norA efflux pump in SaCHG clearly show that continuous exposure
to low concentrations of biocides can activate efflux mechanisms which may in turn contribute to increased antibiotic resistance. The results confirm previous findings in section 3.4, where efflux pump inhibitors thioridazine and chlorpromazine successfully increased the susceptibility of oxacillin resistant strain SaCHG. It also show that efflux pump inhibitors potentiate the effects of antimicrobials against tolerant bacteria (Kristiansen and Amaral 1997; Cohn and Rudzienski 1997; Wainwright et al. 1998; Amaral and Kristiansen 2001 Viveiros and Amaral 2001).

The data presented suggest efflux as one of the survival mechanisms which could be activated during the adaptation process, when bacterial strains were exposed to sub-inhibitory concentrations of biocides.

To further confirm that efflux mechanism did contribute to the antibiotic resistance observed and to see any antibiotic resistant genes were involved, quantitative Realtime PCR was used to compare expression levels of selected antibiotic resistant genes between wild type and antibiotic resistant strains. The data obtained in section 4.3.3 showed that the outer membrane porin gene ompF was significantly downregulated in EcH2O2 and norA efflux pump was significantly upregulated in SaCHG after both were primed with sub-inhibitory concentrations of hydrogen peroxide and chlorhexidine respectively. These results show that non-specific protective mechanisms such as efflux and porins can be activated by low concentration of biocides to cause antibiotic cross-resistance in bacteria (Costa et al. 2013; Bogomolnaya et al. 2013; Jaffe et al. 1982; Manzoor et al. 1999).
The findings agree with previous work showing that biocidal targets (since the bacteria were originally exposed to biocides) within bacteria are non-specific (Maillard 2002; Russell 2002). It also shows that common mechanisms of cross-resistance with antibiotic involves non-specific processes such as efflux mechanisms (Costa et al. 2013; Bogomolnaya et al. 2013), modification of cell wall and cell membrane proteins (Jaffe et al. 1982; Manzoor et al. 1999). An example of which is the reduced permeability in Gram-negative bacteria via the downregulation of porins such as E. coli ompF gene (seen in section 4.3.3) which reduces uptake of antibiotics such as β-lactams (Harder et al. 1981; Pages et al. 2008; Davin-Regli et al. 2008; James et al. 2009).

6.4. Fitness cost of antibiotic resistance

Previous work shows that mutations that confer resistance in bacteria may come with large fitness costs that can affect their growth and replication rates in environment without the selective agent. Costs that will allow them to be outcompeted by susceptible strains in the population (Gillespie and McHugh 1997; Andersson and Levin 1999).

The results of the growth assays between wild type and antibiotic resistant strains show that, although there may be fitness cost associated with antibiotic resistance, bacteria sometimes have trade-offs and other beneficial mutations that compensates for or offset the imbalance that may occur from a selective advantage (Olivares 2014; Knopp and Andersson 2015; Phan and Ferenci 2017). However, these trade-offs and compensatory mutations can sometimes mean reversing the antibiotic resistant phenotype (Knopp and Andersson 2015), although not observed in the present study.
but shown in the work of Knopp et al. where antibiotic sensitivity was restored due to overly expressed *phoE* in *ompFC* deficient *E. coli* after several generations of tolerance (Knopp and Andersson 2015).

When grown in the absence of antibiotic, resistant strains are expected to be outcompeted by susceptible strains (Levin *et al.* 1997; Johnsen *et al.* 2009; Melnyk *et al.* 2015), which did not happen in this study. The data obtained here however supports clinical and epidemiological studies where reducing or completely removing antibiotics does not always eliminate resistant strains (Enne *et al.* 2001), although it will be interesting to see if this happens to the mutants produced in this study when they are continuously sub-cultured without antibiotics.

The observed significant reduced expression of *ompF* porin gene in β-lactam resistant EcH2O2 and the significant expression of *norA* efflux gene in oxacillin resistant SaCHG was more reason to expect large fitness costs in these strains, this was however not observed.

While there are no observable fitness costs in the comparative assays carried out in this study, it will be good to bear in mind that these conditions were *in vitro* therefore, results from *in vivo* conditions were animal models are used such as nematode killing may differ (Sánchez *et al.* 2002; Pope *et al.* 2010).
6.5. Conclusion

Public awareness to infection control, for example the present day COVID-19 pandemic can contribute to the uncontrolled wide-spread use of biocide and biocidal products especially at below in-use concentrations. This is a cause for concern because the use of any antimicrobial at sub-inhibitory concentrations can create selective pressure that will favour the development of adaptive strains (McBain and Gilbert 2001; Fraise 2002; SCENIHR 2010; Maillard et al. 2013 Knapp et al. 2015).

While adaptive resistance is understood to be a non-genetic response and a temporary survival tactics employed by bacteria (Erickson et al. 2017), the divergence that arises from the adaptive process may involve both genetic (Tenaillon et al. 2012; Toprak et al. 2012) and non-genetic mechanisms (Fong and Palsson 2005; Adam et al. 2008; López-Maury et al. 2008).

6.5.1. The data presented in this study suggests that;

- Prolonged exposure of bacteria to low concentrations of biocide can prime bacteria to become resistant to antibiotics even in the absence of increased tolerance to the biocide.

- Continuous exposure to low concentrations of biocides may activate efflux mechanisms which can in turn contribute to increased tolerance to biocide and cross-resistance to antibiotics.

- Efflux pump inhibitors at sub-inhibitory concentrations, can increase the susceptibility of resistant bacteria to antibiotics.
• Sub-inhibitory use of biocides may trigger the activation of protective response in bacteria that can lead to biocide tolerance and antibiotic cross-resistance.

• Fitness cost associated with antibiotic resistance may not always be apparent and can be reduced through natural selection.

This study reinforces the knowledge that the widespread use of biocides especially at below in-use concentrations may exacerbate the ongoing antibiotic resistance in clinically relevant bacteria (Gilbert et al. 2002; SCENIHR 2009; SCENIHR 2010) and has shown that sub-inhibitory use of biocides may trigger the activation of protective response genes in bacteria that can lead to increased antibiotic resistance.

These results show that the use of low concentrations of biocides, especially in formulated products such as the HIBI hand rub used in this study which contained just 0.5 % chlorhexidine can activate antibiotic resistance mechanisms. A previous review found that various formulated products such as mouth wash, catheter cleaning solutions and wound dressing being used by the National Health Service (NHS) contains chlorhexidine concentrations ranging from 0.02 % to 4 % (Wand et al. 2016). This is a serious cause from concern knowing the effect of low concentrations on selective pressure as observed in this study.

The results observed in this study also show that not all antibiotic resistant phenotype comes with large or observable cost to fitness, bacteria sometimes have trade-offs and other beneficial mutations that compensates for or offset imbalances that may occur from a selective advantage (Olivares et al. 2014; Knopp and Andersson 2015; Phan and
Ferenci 2017). Even if these trade-offs and compensations sometimes reverses resistant phenotypes (Knopp and Andersson 2015), although not observed in this present study, but was in the work of Knopp et al., where antibiotic sensitivity was restored due to overly expressed *phoE* in *ompFC* deficient *E. coli* after several generations of tolerance (Knopp and Andersson 2015).

The fact that the antibiotic resistance and changes in gene expression did not cause observable significant fitness cost in the strains tested here, highlight the challenges that could be faced in any clinical setting, where biocides are used for infection control purposes.

While there are no observable fitness cost in the competitive assays carried out in this study, results from *in vivo* conditions where animal models are used such as nematode killing may vary (Sánchez et al. 2002; Pope et al. 2010).

6.6. Recommendations

The findings of this study highlight the downside of biocide misuse, although only two-fold tolerance to one biocide (benzalkonium chloride) was observed out of the four biocides studied. However, the cross-resistance to antibiotics in the absence of stable increased tolerance to the other biocides is worrying. Strict guidelines need to be put in place to inform consumers of the potential risks involved in biocide misuse. Especially in the current COVID-19 pandemic where the use of surface disinfectants, hand sanitisers and antimicrobial soaps (some containing biocides) have increased in both health care and community settings (Murray 2020). The potential risk of sub-inhibitory
concentration of biocide entering wastewater plants and causing selective pressure in resident microbial population has increased, with the increase use of disinfectant, hand sanitiser and soap containing biocides (Berardi et al. 2020; European Parliament 2020; Hegarty 2020; Murray 2020).

Although there is surveillance in place for the control of antibiotics use (Gould et al. 1994 Shlaes et al. 1997, Zarb et al. 2012, Goldmann et al. 1996) and some regulations requiring manufacturers of biocides and formulated products to show evidence that the use of their products may not cause antimicrobial tolerance (Biocidal Product Regulation 2012; Knapp; et al. 2015). It is now important more than ever to understand and acknowledge the impact of biocide use and misuse on the development of biocide tolerance and antibiotic cross-resistance in clinically relevant bacteria. The origin, evolution and mechanisms of resistance involved needs to be understood in order the tackle the issues presented in this study.

6.7. Limitations of study

Ethidium bromide efflux assay and β-lactamase test were used to test efflux pump activity and production of β-lactamase enzymes, in antibiotic resistant strains using wild type strains as negative control. The use of known efflux pump expressing and β-lactamase producing strains would have provided more robust comparisons.
6.8. Future work

While this work has answered questions on the effect of low concentration of biocides on antibiotic resistance and the mechanisms that may be involved, it would be interesting to see the real-time response in protective mechanisms such as the *soxRS* and *oxyR* operons during stepwise exposure to biocides such hydrogen peroxide by carrying out gene expression analysis immediately after each stage of exposure. The extent of activation of efflux mechanisms such as *acrA* and downregulation of porins such as *ompF* will be studied by conducting gene expression at varying concentrations of the antibiotics that are resistant too.

In order to identify other mechanisms that may be contributing to the antibiotic cross-resistance, gene knock-out experiment will be carried, it will be interesting to see if β-lactam resistant strain EcH2O2 will become more susceptible to the antibiotics if the *ompF* gene is knocked out or if another gene will be downregulated instead. The SaCHG *norA* efflux gene will also be knocked out to see if resistance to oxacillin will still occur.

The effect of constant sub-culture on the antibiotic resistance of EcH2O2, SaCHG and PaGTA will be tested, to see if they completely loss their resistance and revert to wild-type or become more resistant. Quantitative Realtime PCR will also be used to test changes in *ompF* and *norA* expression in regularly sub-cultured strain compared the strains stored at -80°C.
Since no mechanism of resistance was identified in ciprofloxacin resistant strain PaGTA, more antibiotic resistant genes such the *P. aeruginosa* *oprD* porin gene will be tested for changes in nucleotide sequence as well expression levels.
7. References


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Table 8.1 Accession numbers of *Escherichia coli* gene primers used in this study

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>acrA</em></td>
<td>F/ CAGTAAGCAAGAGTACGATCAGG</td>
<td>CP022959.1:3455926-3457119</td>
</tr>
<tr>
<td><em>acrA</em></td>
<td>R/ GGAGAGGTGACTTTGGTGTAAG</td>
<td></td>
</tr>
<tr>
<td><em>acrB</em></td>
<td>F/ GTCGATTCGTTCTCCTCATTG</td>
<td>CP022959.1:3457142-3460291</td>
</tr>
<tr>
<td><em>acrB</em></td>
<td>R/ ACCTGGAAGTAACGCTATTGG</td>
<td></td>
</tr>
<tr>
<td><em>soxR</em></td>
<td>F/ GGCATACTTAAGTGCGAAAG</td>
<td>CP022959.1:4356618-4357082</td>
</tr>
<tr>
<td><em>soxR</em></td>
<td>R/ ACCAATAACATCCGTTCAGTTC</td>
<td></td>
</tr>
<tr>
<td><em>soxS</em></td>
<td>F/ GCCGCTTAACATTGATGATGTC</td>
<td>CP022959.1:4357168-4357491</td>
</tr>
<tr>
<td><em>soxS</em></td>
<td>R/ GCCGTTGGCGAATGTAATC</td>
<td></td>
</tr>
<tr>
<td><em>marA</em></td>
<td>F/ AATACATCGCGAGCCGTAAAG</td>
<td>CP022959.1:2324954-2325619</td>
</tr>
<tr>
<td><em>marA</em></td>
<td>R/ TTGCGACTCGAGGCATATC</td>
<td></td>
</tr>
<tr>
<td><em>marC</em></td>
<td>F/ GGCTTAATCGCGGTCTATTAC</td>
<td>CP022959.1:2324954-2325619</td>
</tr>
<tr>
<td><em>marC</em></td>
<td>R/ GGCTCATCTTTCCAGCTCTT</td>
<td></td>
</tr>
<tr>
<td><em>marR</em></td>
<td>F/ AGTTTAAGGTTGCTCTTATCTC</td>
<td>CP022959.1:2324309-2324743</td>
</tr>
<tr>
<td><em>marR</em></td>
<td>R/ CGTTCAACCCGACCTTTTA</td>
<td></td>
</tr>
<tr>
<td><em>oxyR</em></td>
<td>F/ TTGAGTACCTGGTGCCATTG</td>
<td>CP022959.1:4473637-4474284</td>
</tr>
<tr>
<td><em>oxyR</em></td>
<td>R/ ATCACCAGCAACTCATCTTC</td>
<td></td>
</tr>
<tr>
<td><em>sodA</em></td>
<td>F/ TCCGCTCCGTGAGTAATCTC</td>
<td>CP022959.1:4534570-4535190</td>
</tr>
<tr>
<td><em>sodA</em></td>
<td>R/ GTAGAAAACCACCGCGATTGA</td>
<td></td>
</tr>
<tr>
<td><em>ompF</em></td>
<td>F/ GCGBAATACCGGTTGATTTCC</td>
<td>CP000946.1:2923736-2924464</td>
</tr>
<tr>
<td><em>OmpF</em></td>
<td>R/ ACCAGATCAGATCAGAGATCAG</td>
<td></td>
</tr>
</tbody>
</table>
**Table 8.2** Accession numbers of *Pseudomonas aeruginosa* gene primers used in this study

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MexJ</em></td>
<td>F/ CCTTTCCAGGCGAGATCC</td>
<td>NZ_AYUC01000059.1:170886-171989</td>
</tr>
<tr>
<td><em>MexJ</em></td>
<td>R/ GGGCTGGTCCTTCTTTACC</td>
<td>NZ_AYUC01000059.1:170886-171989</td>
</tr>
<tr>
<td><em>MexL</em></td>
<td>F/ AAGGTCCTGCTGAATATCGC</td>
<td>NZ_AYUC01000059.1:170152-170790</td>
</tr>
<tr>
<td><em>MexL</em></td>
<td>R/ TCGAAGAACAGCTCGAAGG</td>
<td>NZ_AYUC01000059.1:170152-170790</td>
</tr>
</tbody>
</table>

**Table 8.3** Accession numbers of *Staphylococcus aureus* gene primers used in this study

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>norA</em></td>
<td>F/ GGTGGATGAGTGCTGGTATG</td>
<td>NZ_CP020020.1:715029-716195</td>
</tr>
<tr>
<td><em>norA</em></td>
<td>R/ TCGCTGACATGTAGCCAAAAG</td>
<td>NZ_CP020020.1:715029-716195</td>
</tr>
<tr>
<td><em>lmrS</em></td>
<td>F/ CTACATCGCCATCGCATTAAAC</td>
<td>NZ_CP020020.1:2211577-2213019</td>
</tr>
<tr>
<td><em>lmrS</em></td>
<td>R/ GCTATCACAAGCTGCGATAAC</td>
<td>NZ_CP020020.1:2211577-2213019</td>
</tr>
</tbody>
</table>
Table 8.4 Accession numbers of housekeeping gene primers used in this study

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Sequence</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gap</em></td>
<td>F/ CGACCTGTTAGACGCTGATTAC</td>
<td>CP022959.1:2052919-2053914</td>
</tr>
<tr>
<td><em>Gap</em></td>
<td>R/ CGATCAGATGACCGTCTTTCAC</td>
<td></td>
</tr>
<tr>
<td><em>rhO</em></td>
<td>F/ ACAATCCGGGTGTTGTTAAG</td>
<td>CP022959.1:4663008-4664267</td>
</tr>
<tr>
<td><em>rhO</em></td>
<td>R/ GTACAGCGTGCTGATTGT</td>
<td></td>
</tr>
<tr>
<td><em>ftsZ</em></td>
<td>F/ GCTGCTGAACCTGCTTATTG</td>
<td>CP022959.1:3893110-3894261</td>
</tr>
<tr>
<td><em>ftsZ</em></td>
<td>R/ GTACTTCTCTTGACCCGATATG</td>
<td></td>
</tr>
<tr>
<td><em>recF</em></td>
<td>F/ ACCGATTCATGCTGATC</td>
<td>CP043920.1:4264-5376</td>
</tr>
<tr>
<td><em>recF</em></td>
<td>R/ AGTCGTGCCGCTTCATT</td>
<td></td>
</tr>
<tr>
<td><em>secA</em></td>
<td>F/ CGAAGGAAGGCCTCAATTTC</td>
<td>CP043302.1:341170-343701</td>
</tr>
<tr>
<td><em>secA</em></td>
<td>R/ CTGTTCATCTCCGCAAGTTCATT</td>
<td></td>
</tr>
<tr>
<td><em>recA</em></td>
<td>F/ CGCCGAAGCATTTGGTAGAG</td>
<td>CP043914.1:1278511-1279554</td>
</tr>
<tr>
<td><em>recA</em></td>
<td>R/ ACGTGAGTGCTCCCATTTC</td>
<td></td>
</tr>
<tr>
<td><em>recA</em></td>
<td>F/ CAACGTCCGGTGTCCTTCATC</td>
<td>LR700248.1:5432386-5433426</td>
</tr>
<tr>
<td><em>recA</em></td>
<td>R/ CGTAGGAACCTCAGTGCGTTACC</td>
<td></td>
</tr>
<tr>
<td><em>adK</em></td>
<td>F/ CGCCTGTCGCTATCATCATT</td>
<td>LR700248.1:5357405-5358052</td>
</tr>
<tr>
<td><em>adK</em></td>
<td>R/ CGGGCGATGCTGTTATTT</td>
<td></td>
</tr>
</tbody>
</table>
8.1. Determination of housekeeping genes

Housekeeping genes used in this study were selected based on previous validation and use in previous studies (Rocha et al. 2015) geNorm version 3.5 was used to determine and select stably expressed housekeeping/reference genes or gene pair across sample conditions (Vandesompele et al. 2002) Figure 8.1 (a), for *E.coli* strains glyceraldehyde-3-phosphate dehydrogenase (*gap*) and cell division protein gene *ftsZ* were determined to be stable together, Figure 8.1 (b), for *S. aureus* strains, recombinase A gene (*recA*) and DNA replication and repair protein gene *recF* was both used for normalization and, Figure 8.1 (c), for *P. aeruginosa* recombinase A (*recA*) and adenylate kinase (*adk*) were used for normalization shows validated housekeeping genes used in this study.
GeNorm version 3.5 was used to determine and select stably expressed housekeeping genes or gene pair across sample conditions (Vandesompele et al. 2002). (a) *E.coli* glyceraldehyde-3-phosphate dehydrogenase (gap) and cell division protein gene ftsZ were determined stable together for *E.coli* so they were both used to normalize Cq values of samples, (b) recombinase A gene (recA) and DNA replication and repair protein gene recF was both used for *S.aureus* and (c) recombinase A (recA) and adenylate kinase (adK) were used for normalization *P. aeruginosa*.

**Figure 8.1.** Validation of housekeeping genes using geNorm
8.1.2. Gene efficiency

To determine gene and primer efficiency, genomic DNA templates for standard curves were quantified and diluted serially in 5 dilutions of 1:10. Triplicate of each template dilutions were used to give greater confidence in data. Acceptable range of efficiency is between 90 and 110 %. Table 8.1 shows data of gene and primer efficiency used in this study.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Efficiency (%)</th>
<th>R2</th>
<th>Slope</th>
<th>y-intercept</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrA</td>
<td>101</td>
<td>0.99</td>
<td>-0.304</td>
<td>2.99</td>
<td>$y = -0.305 \times x + 2.995$</td>
</tr>
<tr>
<td>acrB</td>
<td>95</td>
<td>0.99</td>
<td>-0.29</td>
<td>3.08</td>
<td>$y = -0.292 \times x + 3.081$</td>
</tr>
<tr>
<td>soxR</td>
<td>102</td>
<td>0.99</td>
<td>-0.30</td>
<td>3.40</td>
<td>$y = -0.307 \times x + 3.410$</td>
</tr>
<tr>
<td>soxS</td>
<td>90</td>
<td>0.99</td>
<td>-0.27</td>
<td>4.53</td>
<td>$y = -0.279 \times x + 4.540$</td>
</tr>
<tr>
<td>ompF</td>
<td>91</td>
<td>0.99</td>
<td>-0.28</td>
<td>2.60</td>
<td>$y = -0.282 \times x + 2.610$</td>
</tr>
<tr>
<td>micF</td>
<td>108</td>
<td>0.99</td>
<td>-0.32</td>
<td>3.08</td>
<td>$y = -0.319 \times x + 3.082$</td>
</tr>
<tr>
<td>marC</td>
<td>90</td>
<td>0.99</td>
<td>-0.28</td>
<td>2.87</td>
<td>$y = -0.280 \times x + 2.878$</td>
</tr>
<tr>
<td>marR</td>
<td>90</td>
<td>0.99</td>
<td>-0.27</td>
<td>2.52</td>
<td>$y = -0.279 \times x + 2.524$</td>
</tr>
<tr>
<td>marA</td>
<td>92</td>
<td>0.99</td>
<td>-0.28</td>
<td>2.70</td>
<td>$y = -0.284 \times x + 2.705$</td>
</tr>
<tr>
<td>oxyR</td>
<td>91</td>
<td>0.99</td>
<td>-0.28</td>
<td>4.36</td>
<td>$y = -0.282 \times x + 4.366$</td>
</tr>
<tr>
<td>sodA</td>
<td>91</td>
<td>0.99</td>
<td>-0.28</td>
<td>2.76</td>
<td>$y = -0.281 \times x + 2.766$</td>
</tr>
<tr>
<td>Gap</td>
<td>101</td>
<td>0.99</td>
<td>-0.30</td>
<td>2.86</td>
<td>$y = -0.303 \times x + 2.869$</td>
</tr>
<tr>
<td>ftsZ</td>
<td>94</td>
<td>0.99</td>
<td>-0.28</td>
<td>2.61</td>
<td>$y = -0.289 \times x + 2.618$</td>
</tr>
<tr>
<td>rhO</td>
<td>104</td>
<td>0.99</td>
<td>-0.31</td>
<td>2.87</td>
<td>$y = -0.310 \times x + 2.875$</td>
</tr>
<tr>
<td>secA</td>
<td>100</td>
<td>0.99</td>
<td>-0.30</td>
<td>3.5</td>
<td>$y = -0.303 \times x + 3.510$</td>
</tr>
<tr>
<td>recF</td>
<td>100</td>
<td>0.99</td>
<td>-0.30</td>
<td>3.2</td>
<td>$y = -0.301 \times x + 3.234$</td>
</tr>
<tr>
<td>recA</td>
<td>99</td>
<td>0.99</td>
<td>-0.29</td>
<td>3.2</td>
<td>$y = -0.299 \times x + 3.200$</td>
</tr>
<tr>
<td>norA</td>
<td>96</td>
<td>0.99</td>
<td>-0.29</td>
<td>3.2</td>
<td>$y = -0.293 \times x + 3.227$</td>
</tr>
<tr>
<td>lmrS</td>
<td>104</td>
<td>0.99</td>
<td>-0.3</td>
<td>3.5</td>
<td>$y = -0.311 \times x + 3.557$</td>
</tr>
<tr>
<td>recA</td>
<td>101</td>
<td>0.99</td>
<td>-0.3</td>
<td>4.5</td>
<td>$y = -0.304 \times x + 4.598$</td>
</tr>
<tr>
<td>adK</td>
<td>105</td>
<td>0.99</td>
<td>-0.3</td>
<td>3.7</td>
<td>$y = -0.313 \times x + 3.776$</td>
</tr>
<tr>
<td>mexL</td>
<td>101</td>
<td>0.99</td>
<td>-0.3</td>
<td>5</td>
<td>$y = -0.304 \times x + 5.075$</td>
</tr>
<tr>
<td>mexJ</td>
<td>89.6</td>
<td>0.95</td>
<td>-2.8</td>
<td>5.8</td>
<td>$y = -0.277 \times x + 5.819$</td>
</tr>
</tbody>
</table>
8.1.3. *norA* promoter region

Primers previously designed in chapter 4 (Table 4.1) were used to carry out PCR on the promoter region of SaATCC and SaCHG to identify the presence of *norA* promoter region in both strains. PCR fragments were electrophoresed and **Figure 8.2 (a)**, lane 1 and 2 shows bands corresponding to *norA* promoter primers for SaATCC and SaCHG, expected amplicon size 636 base pairs.

Purified PCR products of SaATCC and SaCHG *norA* promoter region was sequenced and analysed using NACBI BLAST to see if the cross-resistance to oxacillin in SaCHG could be linked to change in nucleotide sequence. **Figure 8.2 (b)** shows there was no change in nucleotide sequence.
Figure 8.2. PCR and sequence analysis of norA promoter region

PCR was carried out on norA promoter region in SaATCC AND SaCHG (a) and (b)

Gene-specific primers were used for each PCR reaction; PCR products were separated by gel electrophoresis on 1% agarose gels. The standard of the molecular weight ladder, 50 bp (lane 1) are shown on the left. Purified PCR products of SaATCC and SaCHG norA promoter region was sequenced and analysed using NACBI BLAST to see if the cross-resistance to oxacillin in SaCHG could be linked to change in nucleotide sequence. shows there was no change in nucleotide sequence. There was no nucleotide change observed.
Figure 8.3 shows a representation of the process involved in the measurement of differential expression of ompF in EcH2O2 grown in 4 mg/l cephalothin, EcATCC was used as control baseline. Figure 8.3 (a) Cq of endogenous controls were calculated using qPCR, Figure 8.3 (b) the Cqs are were normalised using endogenous control genes gap and fitZ (previously validated with geNorm in Figure 8.1) and Figure 8.3 (c) the differential expression of ompF was calculated. Experiments were done in technical and biological triplicates.

Figure 8.3. Process involved in ompF expression

Differential expression of ompF in EcH2O2 grown in 4 mg/l cephalothin, using the EcATCC as control base line. (a) qPCR raw measurement: Cqs of endogenous controls (gap green bars and fitZ blue bars) and ompF were calculated using qPCR, (b) ompF normalised Cqs: the Cqs are were normalised with endogenous control genes gap and fitZ and (c) ompF expression fold-change: the differential expression of ompF was calculated and represented in log 10 scale. (n=3).
The process involved in the measurement of differential expression of norA in SaCHG grown in 0.125 mg/l oxacillin, SaATCC was used as control baseline is shown in Figure 8.4 (a) Cqs of endogenous controls (recA and recF) and norA were calculated using qPCR, Figure 8.4 (b) the Cqs are were normalised using endogenous control genes recA and recF (previously validated with geNorm in Figure 8.1) and Figure 8.4 (c) the differential expression of norA was calculated and represented in log scale. Experiments were done in technical and biological triplicates.

![Figure 8.4. Process involved in norA expression](image)

Differential expression of norA in SaCHG grown in 0.125 mg/l oxacillin, using SaATCC as control baseline. (a) qPCR raw measurement: Cq of endogenous controls were calculated using QRTPCR (recA blue bars and recF green bars), (b) norA normalised Cq: the Cqs are were normalised with endogenous control genes recombinase, A gene (recA) and DNA replication and repair protein gene recF previously validated with geNorm (c) norA expression fold change: the differential expression of norA was calculated and represented in log 10 scale. (n=3; error bars = mean ± standard error of the mean).