Evaluating the role of efflux pumps in bacterial disinfectant resistance

By

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Declaration

I, Gunther Johann Staats, declare that the research dissertation that I herewith submit for the master's degree qualification in Microbiology at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

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Abstract

The global rise of antibiotic resistance could lead to the advent of a post-antibiotic era, where disinfectants and biosecurity will be vital parts to control microbial proliferation. The SARS-CoV-2 pandemic has shown how important proper biosecurity and disinfection protocols are to control disease outbreaks. Disinfectant resistance has the potential to alter every aspect of disease control, as this phenomenon impacts human life from food security to healthcare systems. Antimicrobial resistance at its core originates from the presence and regulation of specific genes within the genome of a microorganism able to combat/resist specific action of an antimicrobial. This study focuses on investigating whether specific resistance determinants are responsible for the insusceptibility of a highly resistant isolate, *Serratia* sp. HRI. This isolate has high levels of disinfectant resistance; therefore, it provides an opportune chance to study if a specific mechanism is responsible. To achieve this the genomes of the *Serratia* sp. HRI and its closest related type strain were investigated for efflux pump genes. The efflux pump genes were predicted using an automatic annotation pipeline. The predictions revealed a plethora of resistance efflux genes mostly harbouring multidrug functioning. Additionally, disinfectant-specific efflux pump genes were identified (*emrE*, *sugE*, *qacA*, *qacE*, and *ssmE*). Susceptibility testing using three disinfectants revealed how the resistance levels between the two *Serratia* isolates differed. Further investigation using efflux pump inhibitors (EPIs) showed how specific families of efflux pumps confer resistance in both isolates. Time-kill analyses over an extended period showed how *Serratia* sp. HRI tolerates long-term disinfection. Using EPI reserpine, the efflux pump activity was determined during long-term disinfection. The results showed that concentration-dependent recruitment of efflux pumps was seen by *Serratia* sp. HRI. At low disinfectant concentrations, another mechanism was responsible for the survivability of the bacteria. Using liquid chromatography with tandem mass spectrometry it was established that disinfectant levels introduced with *Serratia* sp. HRI decreased over time. Suggesting that the HRI isolate has some mechanism to alter the structure of the disinfectant,

such as metabolism or degradation pathways. This work highlights the role of efflux pumps in disinfectant resistance and the potential of other mechanisms to be involved. Future research will include gene deletion and expression studies to fully determine the efflux pump reliance for disinfectant resistance. The work completed in this thesis added to the knowledge of efflux-mediated disinfectant resistance. This work also highlighted the potential role of metabolism/degradation in resistance to low-level disinfection.

List of Abbreviations

BAC: Benzalkonium chloride, alkyldimethylbenzylammonium chloride

DDAC: Didecyldimethylammonium chloride

MIC: Minimum Inhibitory Concentration

EPI: Efflux Pump Inhibitor

QAC: Quaternary Ammonium Compound

RSP: Reserpine

CCCP: Carbonyl Cyanide 3-Chlorophenylhydrazone

LC: Liquid Chromatography

MS: Mass Spectrometry

LC-MS/MS: Liquid Chromatography with Tandem Mass Spectrometry

MDR: Multidrug Resistance

HRI: Highly Resistant Isolate

TransAAP: Transporter Automatic Annotation Pipeline

ROS: Reactive Oxygen Species

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SMR subfamily forms part of the DMT superfamily and the MATE subfamily forms part of the MOP superfamily. The PACE family is a newly characterised (Verma et al. 2021).

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[Publication] Figure 1. Total transporter predictions of *Serratia* isolates into five main superfamilies of efflux pumps. The *p*-value of the chi-square test > 0.05; therefore, the *Serratia* isolates, and occurrence frequency of efflux pump superfamilies are independent.

[Publication] Figure 2. Resistance transporter predictions of *Serratia* isolates into various efflux families. The *p*-value of the chi-square test > 0.05; therefore, the *Serratia* isolates, and occurrence frequency of efflux pump families are independent.

[Publication] Figure 3. Mean disinfectant concentration of the MIC of *Serratia* isolates. Bars: geometric mean ± 95% C.I. as error bars (*n* = 3 biological replicates). Significance of difference to type strain indicated by asterisks: **p* < 0.0001 (two-tailed unpaired *t*-test of disinfectant concentrations).

[Publication] Figure 4. Mean disinfectant concentration of MIC for *S*. sp. HRI with supplementation of EPIs RSP at 5 µg/mL and CCCP at 12.5 µM final concentrations. Bars: geometric mean ± 95% C.I. as error bars (*n* = 3 biological replicates). Significance of difference

to HRI isolate with only disinfectant indicated by asterisks: *** $p < 0.001$ (multiple comparison Bonferroni-adjusted ANOVA). Different letters indicate differences to HRI isolate with only disinfectant.

[Publication] Figure 5. Mean disinfectant concentration of MIC for *S. marcescens* subsp. *marcescens* ATCC 13880 with supplementation of EPIs RSP at 5 µg/mL and CCCP at 12.5 µM final concentrations. Bars: geometric mean ± 95% C.I. as error bars (n = 3 biological replicates). Significance of difference to ATCC strain with only disinfectant indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (multiple comparison Bonferroni-adjusted ANOVA). Different letters indicate differences to ATCC isolate with only disinfectant.

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Chapter 1: Literature Review

1.1 Introduction to the current study

Since the inception of quaternary ammonium compounds (QACs), these antimicrobial agents have been widely used in medical, food and veterinary environments for routine disinfection and control of pathogenic bacterial proliferation (Jennings et al. 2015). Outside of these environments, QACs are also integrated into pharmaceutical and domestic products at concentrations below the recommended bactericidal concentration (Gilbert and McBain 2003). The excessive use of QACs leads to downstream selective effects once concentrations are present below bactericidal/inhibitory levels causing bacteria to continually adapt to increasing stress (Hegstad et al. 2010). The advent of QAC resistance should be no surprise. However, the rate at which resistance levels are increasing is alarming. Of particular concern is the effects of pathogenic bacteria becoming multidrug-resistant, where antibiotics are no longer effective to control these pathogens, disinfectants like QACs are potentially the final control option. Therefore, understanding how bacteria resist QACs, a commonly used disinfectant, is vital to unravelling the complex nature of bacterial resistance.

QACs proposedly cause the perturbation of the cytoplasmic and outer membrane lipid bilayers, resulting in the general and progressive leakage of cytoplasmic membrane constituents (Gilbert and Moore 2005). Resistance to QACs is generally conferred by intrinsic efflux pumps or membrane alterations (Piddock 2006). Alternatively, acquired resistance genes, like *qac* genes encoding QAC-specific efflux pumps or multidrug resistance genes can be transferred on mobile genetic elements (Bjorland et al. 2003).

Efflux pumps function to remove toxic compounds from the interior of the cell, thus theoretically the expression of efflux pumps to confer QAC resistance would be ineffective (Zgurskaya and Nikaido 2000). The bacterial cell would not benefit from expressing proteins that function to

expel chemicals that are not entering the cell to cause damage. QAC compounds could be entering the bacterial cell through conduit points such as porins, once inside they can cause similar inner membrane disruption while also targeting other important sites, such as DNA. Deciphering how efflux pumps are such effective resistance mechanisms against QACs and how it relates to their mode of action is, therefore required and will be the focus of this study.

1.2 Aims and objectives

This study aims to evaluate whether efflux pumps are involved in conferring disinfectant resistance in *Serratia* sp. HRI. This will be done by comparison of *Serratia* sp. HRI to the closest related type strain, *Serratia marcescens* subsp. *marcescens* ATCC 13880, by susceptibility testing. The hypothesis for this work is that *Serratia* sp. strain HRI possesses efflux pumps which confer high-level disinfectant resistance.

To determine the activity of efflux pumps involved in disinfectant resistance in *Serratia* sp. HRI, two main areas focused upon will be short-term and long-term disinfection. Firstly, short-term disinfection will determine if efflux pumps contribute to the disinfectant tolerance capabilities of *Serratia* sp. HRI during a standard disinfection exposure time. Secondly, long-term disinfection will be used to elucidate efflux pump activity for an extended contact time.

The first objective is to determine the role of efflux pumps in short-term disinfection using QACs and a hydrogen peroxide-peracetic acid disinfectant. This work is presented in the published article in Chapter 2 and includes efflux pump predictions using a database pipeline and minimum inhibitory concentration determinations with and without the addition of efflux pump inhibitors (EPIs). The second objective is to determine efflux pump involvement in long-term disinfection using a QAC. This work is presented in Chapter 3, which includes time-kill assays of various QAC concentrations over an extended period and liquid chromatography

with tandem mass spectrometry (LC-MS/MS) investigation into QAC metabolism. This work is followed by Chapter 4, a general discussion, and conclusions.

1.3 Introduction

Multidrug resistance (MDR) in bacterial populations has increased within the last decade (Klemm et al. 2018; Lopes et al. 2019). The prevalence of antibiotic resistance may lead to a post-antibiotic era and has been described as an emergent global threat (Roca et al. 2015). Currently, one of the best methods to combat unwanted bacterial growth is disinfection techniques and good biosecurity (Bragg et al. 2018). Disinfectants are widely utilised in many settings including domestic, agricultural, food-processing, healthcare, and pharmaceutical environments (Jennings et al. 2015; Kim et al. 2018). These agents can inhibit the proliferation and spread of pathogenic organisms (Chapman 2003). This includes bacteria and viruses in the transmission medium, as evidenced by the use of disinfection protocols during the SARS-Cov-2 pandemic (Chaturvedi et al. 2021). This makes the rapid emergence of disinfectant resistance rather alarming (Bragg et al. 2014). The cause of the emergence can be linked to misuse and abuse of disinfectants, particularly in conjunction with the lack of adequate management and scientific understanding of disinfectants (Campoccia et al. 2010). Additionally, the use of disinfectants has been linked to the increasing prevalence of antibiotic-resistance genes (Bragg et al. 2014; Roca et al. 2015; Kim et al. 2018). However, the concern rises that bacteria are being challenged by subinhibitory levels of disinfectants in environments, which can also lead to the development of resistance to disinfectants and antibiotics simultaneously (Mc Cay et al. 2010; Ribič et al. 2020).

The presence of any antimicrobial agent imposes selective pressure on the bacterial population, which can increase the tolerance of organisms (Ribič et al. 2020). Exposure to antimicrobials can trigger very complex reactions altering levels of expression of various

genes, including transporter genes (Nichols et al. 2012). Phenotypic adaptations, genetic mutations, and horizontal gene transfer (HGT) are methods by which bacteria can increase tolerance (Cloete 2003). Human and general ecosystem health are in jeopardy because of the serious threat that MDR bacteria and MDR genes pose (Cazares et al. 2020). These resistant populations reduce the killing efficacy of disinfectants and antibiotics, which can increase the possibility of resistance transfer between populations (Zhu et al. 2021).

To this end, in 2014 an estimated 700,000 deaths were attributed to antimicrobial resistance and this value was projected to increase to 10 million by 2050 (O'Neill 2016). In 2015, the European Union estimated that 671,689 infections were caused by antibiotic-resistant bacteria, where 63,5% were hospital-acquired. These infections resulted in 33,110 deaths (Cassini et al. 2019). Further, the Centre for Disease Control and Prevention in a 2019 report estimated that roughly 2,5 million bacterial infections occurred in the United States, where roughly 45,000 people died from antibiotic-resistant infections (CDC 2019). Currently, roughly 80% of severe bacterial infections are caused by multidrug-resistant Gram-negative bacteria (Fraimow and Tsigrelis 2011; Viale et al. 2015).

Gram-negative bacteria contain a cell envelope comprising two membranes that function as a barrier to reduce the entry of antimicrobial agents, such as disinfectants and antibiotics (Poole 2002). During the disinfectant challenge, bacteria attempt to reduce the concentration of disinfectant present at the surface of the cell. This is primarily achieved either through biofilm production or reduced membrane permeability (Zhang et al. 2019; Vergalli et al. 2020). If the disinfectant can penetrate the bacterial cell membrane, an oxidative stress response is triggered. This produces large quantities of reactive oxygen species (ROS), which have a synergistic effect on the disinfectant action and bactericidal properties (Li et al. 2016). Generally, the SOS response is enrolled to repair and adapt to reduce damage induced by disinfectant mediated by error-prone DNA repair (Lu et al. 2018). During this period, an

assortment of genes are expressed to assist in the process of adaptation, such as efflux pumps, among others. The efflux pump systems may include either single or a multitude of efflux families able to expel antimicrobial agents (Chapman 2003). However, efflux pumps are not the only genes that can be expressed and can contribute to tolerance capabilities during disinfection. The limited research on the classes of disinfectants and the resultant effect on bacteria is not comprehensive enough under actual conditions. Thus, distinctive clarity on the main resistance mechanisms bacteria use to combat disinfectant exposure and how this links to antibiotic resistance is unclear.

1.4 Quaternary ammonium compounds

QACs are a unique group of compounds that share a common chemical motif, rather than being a single, well-defined substance (Paulson 2002). QACs were first reported to have antibacterial properties in 1916 and were fully introduced as antibacterial defence agents against pathogenic organisms in the 1930's (Jacobs 1916; Russell 2002). These compounds have been used extensively in a wide range of products, ranging from household cleaners and industrial disinfectants to preservatives in eye drops and mouthwashes (Gnanadhas et al. 2013). Additionally, these compounds are even found in some paper processing and wood preservatives (Paulson 2002). QACs are classified as amphoteric surface-active compounds (surfactants). These compounds interact with the cytoplasmic membrane of bacteria, reduce the surface tension and form micelles (Tezel and Pavlostathis 2012). These compounds are also able to interact with intracellular components and bind DNA (Zinchenko et al. 2004). Surfactant compounds are comprised of one hydrocarbon/hydrophobic group and one hydrophilic/polar group (Gilbert and Moore 2005). Furthermore, surfactants can be classified into four groups depending on the charge or absence of ionization, these include cationic, anionic, non-ionic or ampholytic compounds (McDonnell and Russell 1999).

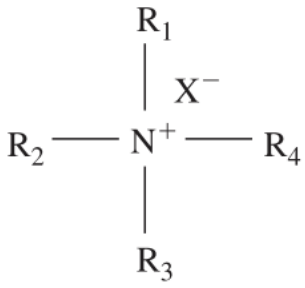

Figure 1.1: The general structure of quaternary ammonium compound. R represents hydrogen atoms, alkyl groups or substituted alkyl groups. X represents an anionic component, such as halides bromide or chloride (Tezel and Pavlostathis 2012).

Structurally, QACs are composed of covalently bound nitrogen (N^+)-containing compounds attached to four other chemical groups (Gilbert and Moore 2005). The general formula is $N^+R_1R_2R_3R_4X^-$, where the R can be a hydrogen atom, alkyl group or alkyl group with additional substitutions and X often represents a halide anion (**Fig. 1.1**) (Hegstad et al. 2010). QACs are mainly comprised of chloride or bromide salts and have a hydrophobic characteristic resulting from the R groups (Gerba 2015). Further, the length of the N-alkyl chain is the main characteristic that determines the antimicrobial capacity of the QACs (Li et al. 2013). Chain lengths of 12- and 16-carbons are most effective in their antibacterial activity (Gerba 2015).

1.5 Antimicrobial activity

QACs are different to antibiotics in that they lack selective toxicity and target specificity, instead having a broad spectrum activity (Jennings et al. 2015). The mechanism responsible for the antimicrobial properties of QACs has not been fully elucidated. Generally, the accepted mechanism involves interaction and subsequent disruption of the cell membrane integrity followed by cellular constituent leakage (Ioannou et al. 2007). However, QACs bactericidal activity has been further extended to include the inactivation of energy-producing enzymes and the denaturation of essential cellular proteins (Ferreira 2015). Additionally, QACs are

potent biofilm eradicators (Jennings et al. 2014). QACs are shown to have greater activity against Gram-positive bacterial species due to them possessing a single phospholipid cellular membrane compared to the dual phospholipid cellular membranes found in Gram-negative bacteria (Jennings et al. 2015). Vegetative bacteria, yeast, moulds, algae, and lipophilic viruses can be eradicated by QACs at higher concentrations. However, bacterial spores, mycobacteria and hydrophilic viruses remain unaffected even at elevated concentrations (Hegstad et al. 2010). Both the bacteriostatic and bactericidal properties of QACs are highly dependent on external environmental factors. Factors such as high microbial density (as in biofilms) or organic load reduced the efficacy of the QACs (Ntsama-Essomba et al. 1997; Hegstad et al. 2010). Chemical factors, such as molecular weight and *N*-alkyl chain length affect the antimicrobial activity of the QACs (Ikeda et al. 1986; Chen et al. 2000; He et al. 2013). Microorganisms have different carbon chain lengths of QACs that are optimal for eradication. For instance, 14 carbons are effective against Gram-positive bacteria, 16 carbons against Gram-negative bacteria and 12 carbons against yeast and filamentous fungi (Gilbert and Moore 2005; Kong et al. 2010). Longer *N*-alkyl chain lengths have greater hydrophobicity enhancing their ability to penetrate the hydrophobic bacterial membrane, essentially acting like a needle bursting a balloon (Murata et al. 2007).

1.6 Mechanism of action

Currently, the main proposed mode of action of QACs against microbial cells is interaction and disruption of cell membranes causing cell leakage (Ioannou et al. 2007). At a molecular level, the net positive charge of QACs promotes the interaction/attraction to the negatively charged head groups of bacterial phospholipid bilayers by ionic interactions. The stronger attraction/interaction of the QACs causes displacement of divalent cations, such as Mg^{2+} and Ca^{2+}, associated with the membrane (Gilbert and Moore 2005). Similar cation displacement capabilities are seen in chelating agents, such as ethylenediaminetetraacetic acid (EDTA)

(Gilbert and Moore 2005). With the anchored head groups facing outward, the hydrophobic tails integrate into the lipid core via hydrophobic interactions (**Fig.1.2**) (Hegstad et al. 2010). These interactions cause rearrangement of the membrane increasing surface pressure in the membrane and decreasing membrane fluidity (Tezel and Pavlostathis 2012). The membrane transitions from a fluid to a liquid crystalline state, foregoing osmoregulatory and physiological functions (Tezel and Pavlostathis 2012). The fragile state of the membrane promotes disintegration and subsequent cell rupture (Gilbert and Moore 2005).

The proposed antimicrobial consequences of QACs are concentration dependent. High concentrations of QACs promote micellar aggregation of bacterial phospholipids and individual QAC molecules. This phenomenon results in cell death resulting from membrane disintegration releasing cytoplasmic contents, coagulated proteins and nucleic acids (**Fig. 1.2**) (Ioannou et al. 2007; Tezel and Pavlostathis 2012). Intermediate QAC concentrations also cause membrane disruption interfering with integral membrane-associated processes such as respiration, solute transport and cell wall biosynthesis (Tezel and Pavlostathis 2012). Low QAC concentrations interrupt the osmotic regulation of the membrane and induce proton leakage by the cationic nature of QAC molecules binding anionic regions (Tezel and Pavlostathis 2012). Furthermore, the cumulative effects of all QAC concentrations affect respiratory enzymes and dissipate the proton motive force (PMF). The interference of the membrane equilibrium alters pathways and reactions associated with microbial metabolism, active transport, oxidative phosphorylation and adenosine triphosphate (ATP) synthesis in the bacteria (Knox et al. 1949; McDonnell and Russell 1999).

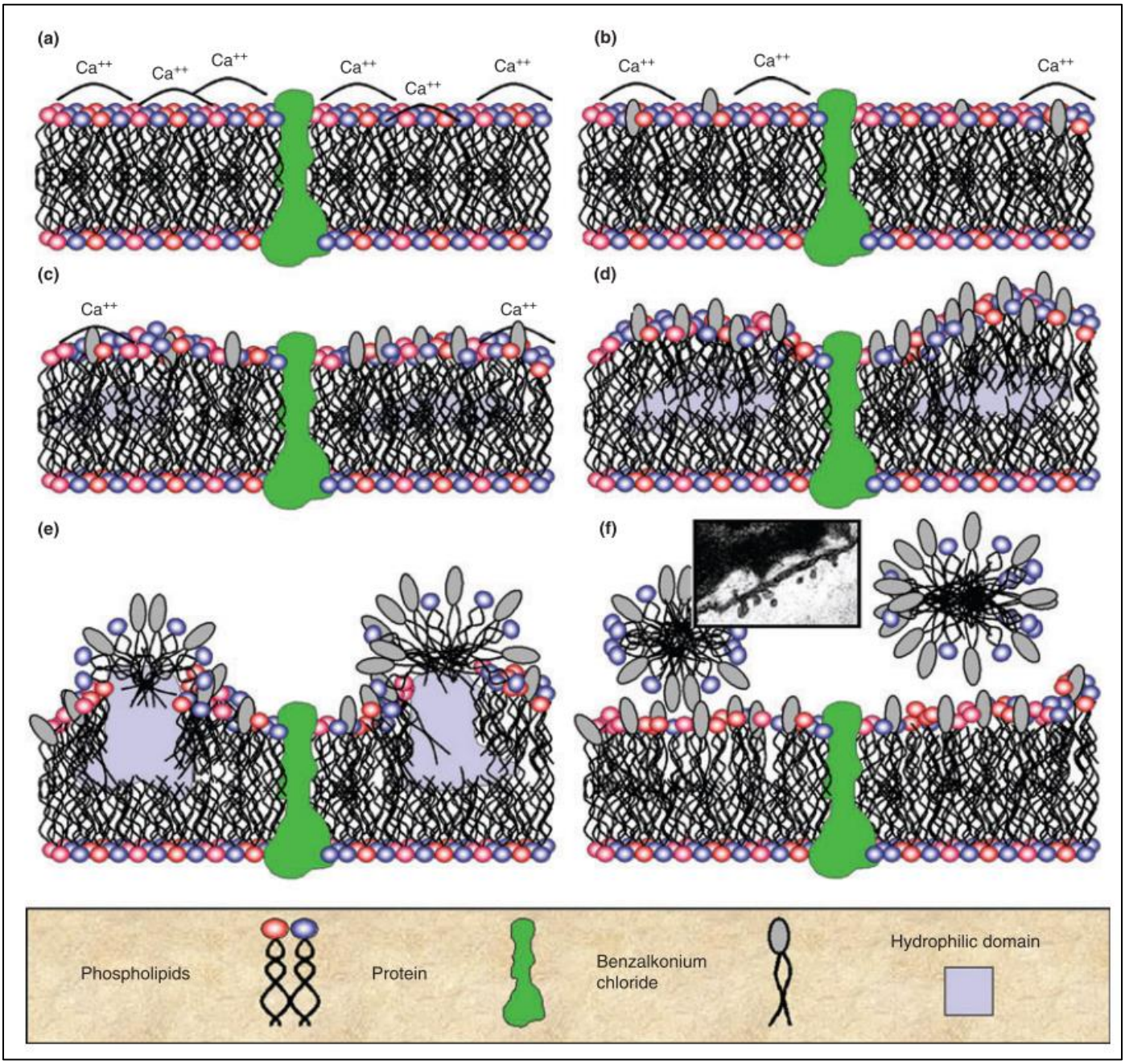

Figure 1.2: Molecular description of QAC mechanism of action. Diagrams (a-f) show the initial interaction and intercalation of the quaternary nitrogen headgroup into the phospholipid layer with increasing QAC concentrations and contact time. The membrane transition from fluid to liquid crystalline promotes hydrophilic voids to emerge within the membrane. The destabilisation of the membrane disrupts protein function because portions of phospholipids and proteins aggregate into QAC-phospholipid micelles. These QAC-phospholipid micelles create large voids within the membrane, leading to eventual cell lysis. The micrograph (f)

shows vesicle formation on the outer membrane during QAC treatment (Gilbert and Moore 2005).

1.7 Bacterial quaternary compound resistance

Considering the mode of action of QACs being non-specific in nature, the development of bacterial resistance would seem unlikely (Gerba 2015). Contrary to the traditional viewpoint that QAC resistance development would be unlikely, clinical reports of QAC-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) and other bacterial isolates have risen in recent years countering this viewpoint (Minbiole et al. 2016). Exposure of bacteria to QACs at subinhibitory concentrations in environments can not only be selected for QAC-resistance development but also antibiotic resistance development (Tandukar et al. 2013; Ortiz et al. 2014). The driving force behind the development of QAC resistance is to prevent the mechanistic action on the membrane of the bacterial cell. The non-specific characteristic of QACs means mutation of a single target will be unlikely to prevent treatment failure (Gerba 2015). QAC resistance mechanisms aim to prevent the interaction of these antimicrobial agents with the membrane or penetration of the cell. Numerous resistance mechanisms are activated, including, physiological membrane alterations and the production of transporter proteins able to expel the QACs (Poole 2005).

1.7.1 Intrinsic resistance

Intrinsic resistance can be described as the tolerance of a species or genus to an antimicrobial chemical based on phenotypic, physiological, or biochemical characteristics (Tezel and Pavlostathis 2012). Membrane permeability barriers, bacterial spores and chromosomally encoded efflux pumps are some of the phenotypic characteristics that bacterial cells can present to confer intrinsic QAC resistance (Hegstad et al. 2010). Specific compositions of the outer membrane and cytoplasmic membrane layers in Gram-negative and Gram-positive

bacteria are highlighted to enhance susceptibility to antimicrobials (Poole 2002). The constituents of bacterial membranes, such as the porin capacity and the lipopolysaccharide (LPS) layer depend on the bacterial phenotype and prevent the antimicrobial activities of antibiotics, QACs and other biocides (Poole 2002). Gram-negative bacteria possessing an outer membrane layer are less susceptible to QACs than Gram-positive bacteria (McDonnell and Russell 1999). Changes in the surface hydrophobicity, outer membrane ultrastructure, protein composition and fatty acid composition allow the outer membrane to contribute to the intrinsic resistance (Poole 2002). The intrinsic resistance against QACs can be further produced by physiological alterations, such as cell walls containing lipids, and stronger LPS-LPS links resulting from high Mg^{2+} content, smaller porins, fewer porins and slime layer development (McDonnell and Russell 1999).

Basal activity levels of chromosomally encoded efflux pumps can confer resistance to QACs (Hegstad et al. 2010). Many of these chromosomally encoded efflux pumps conferring intrinsic resistance to synthetic antimicrobials have physiological functions (Piddock 2006). Chromosomal efflux proteins can transport bacterial substrates, amino acids, ions, and natural substances produced by the infected host, including bile, hormones, and host defensive molecules (Tezel and Pavlostathis 2012). Furthermore, their functionality is extended to aid in colonisation and persistence in the infected host, in the case of food-borne or nosocomial pathogens (Lin et al. 2003; Chen et al. 2013). Often clinically important antibiotics and antimicrobial compounds fall within the multiplicity of substrates that chromosomally encoded efflux pumps can extrude (Tezel and Pavlostathis 2012). In many instances efflux pumps from the resistance-nodulation-division (RND) superfamily are responsible for basal-level QAC resistance, such as SdeXY from *Serratia marcescens*, AcrAB-TolC from *E. coli*, and MexAB-OprM from *Pseudomonas aeruginosa* (Chen et al. 2003; Morita et al. 2003; Randall et al. 2007).

When considering the regulation of efflux pumps the AcrAB-TolC system provides insight into how these proteins are controlled. Global activators (MarA, SoxS, Rob) regulate the expression of the AcrAB-TolC multidrug efflux system. SoxS is an effector of the *soxRS* global superoxide response (*sox*) regulon responsible for the overexpression of AcrAB-TolC (Grkovic et al. 2002). Rob is a binding element to the *E. coli* chromosomal origin of replication (Grkovic et al. 2002). Both SoxS and Rob are homologs of MarA (Grkovic et al. 2002). The *sox* regulon is a group of genes that are responsible for the expression of the AcrAB-TolC efflux system consisting of various genes. The *soxS* gene requires superoxide-generating agents to produce the SoxS protein able to significantly affect the transcription of *acrAB-tolC* (Nunoshiba et al. 1992). This regulon is vital to understand the pathway of resistance to sub-inhibitory QAC concentrations since QACs induce superoxide synthesis at low concentrations (Debbasch et al. 2001). The cell membrane disrupting activity of QACs could interrupt the electron-transport chain resulting in higher levels of ROS within the cell (Bore et al. 2007). The resultant oxidative stress would activate the *soxS* and *marA* effectors leading to the upregulation of *acrAB* and *tolC* gene components encoding a stress-induced efflux pump complex leading to intrinsic resistance (Grkovic et al. 2002; Bore et al. 2007). Concerningly, non-antimicrobial pressures in certain environments can lead to the persistence of antimicrobial resistance. The *soxs* gene was shown to be upregulated by Cu^{2+} in oxidative stress conditions, leading to subsequent AcrAB-TolC expression (Harrison et al. 2009).

1.7.2 Acquired resistance

Prolonged sub-inhibitory exposure of QACs can contribute to the development of clones with lower susceptibility driven by adaptation to the surrounding environment resulting the in evolution and selection of QAC-tolerant bacteria (Gilbert and McBain 2003; Mc Cay et al. 2010). When QACs interact with the bacterial membrane at sub-inhibitory concentrations the bacteria need to compensate for the resultant oxidative stress created and damage to the membrane (Liu et al. 2016). This is generally achieved through the super-oxide scavenging

(SOS)-response and recruitment of stress-response sigma factors (RpoS), which promote mutational events and genetic transfers (**Fig. 1.3**) (Bore et al. 2007; Blázquez et al. 2012). These responses can induce changes in the outer membrane or cytoplasmic membrane, structure and composition, such as porin expression and cell surface hydrophobicity (**Fig. 1.3**) (Tabata et al. 2003; Braoudaki and Hilton 2005). Furthermore, overexpression of efflux pumps and acquisition of extrachromosomal efflux pump genes by mobile recombinant components also contribute to induced changes (**Fig. 1.3**) (Bjorland et al. 2003; Karatzas et al. 2008).

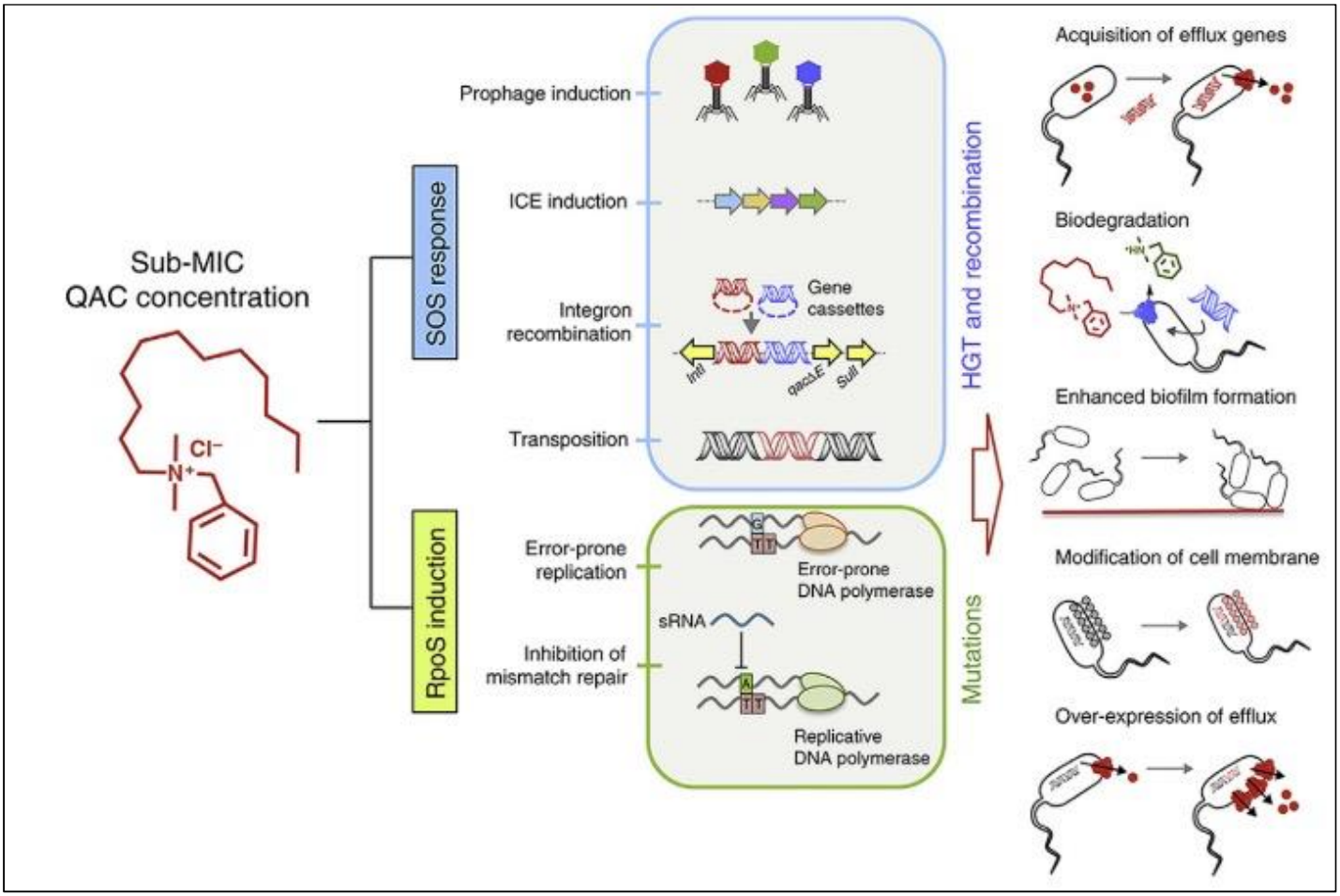

Figure 1.3: Pathways leading to various mechanisms of resistance to QACs. Bacteria can respond to sub-inhibitory concentrations of QACs via the SOS-response and RpoS induction. The induction of either pathway promotes modifications in bacterial cell physiology to

ultimately attain QAC resistance. The modifications resulting from the SOS-response and RpoS induction include QAC biodegradation, enhanced biofilm formation, cell membrane adaptations, over-expression of efflux and acquisition of efflux genes. These modifications are mainly resultant of recombination, HGT or mutational events (Tezel and Pavlostathis 2015).

Various bacterial species can acquire resistance to QACs by altering the permeability of the bacterial cell membrane complex, either both membranes in Gram-negative bacteria or the cytoplasmic membrane in Gram-positive bacteria (Denyer and Maillard 2002). The compositional changes occur by alterations in the fatty acids, phospholipids and outer membrane LPS layer (**Fig. 1.3**) (Boeris et al. 2007). These modifications affect the membrane barriers of bacteria directly resulting in less penetration of QACs through the cell surface (Boeris et al. 2007). The cell surface is changed to a more anionic and hydrophobic nature which restricts the ease with which QACs can pass through the cell surface (Tezel and Pavlostathis 2012). Additionally, porin density and composition, and the protein composition of the bacterial membranes can be changed to hinder QAC interaction and permeation (**Fig. 1.3**) (Ishikawa et al. 2002; Tabata et al. 2003).

Acquired efflux-mediated QAC resistance is a means by which bacteria can combat the intended threat but these proteins often also confer co-resistance to antibiotics (**Fig. 1.3**) (Yamamoto et al. 1988; Wales and Davies 2015). Efflux pumps are proteins situated in the cell membrane able to transfer antimicrobial compounds from the intracellular environment to the extracellular/periplasmic environment using pump-type specific energy mechanisms (Putman et al. 2000). QAC resistance conferred by efflux pumps can either be achieved by overexpression or acquisition of genes via horizontal gene transfer (HGT) (**Fig. 1.3**) (Tezel and Pavlostathis 2012). QAC-induced stress can trigger mutation in regulation systems responsible for efflux pump expression, causing overexpression or increasing extrusion efficiency of specific efflux systems (Grkovic et al. 2002). QAC-induced stress can cause

alterations in efflux pump characteristics that provide tolerance to other unrelated antimicrobials, such as antibiotics, resulting from the large substrate specificity of these transporters (Mc Cay et al. 2010; Tamburro et al. 2015). Acquiring QAC-specific efflux pump genes from integrons, transposons or plasmids introduces new genetic potential for resistance (Bjorland et al. 2003; Li et al. 2017). Many of the efflux pumps introduced by HGT belong to the SMR subfamily, some that are multidrug efflux pumps (SugE) and others that are QAC-specific efflux determinants (QacJ, QacZ) (Bjorland et al. 2003; Braga et al. 2011; He et al. 2011).

Sub-inhibitory QAC exposure has been suggested as a contributor to the evolution and stabilisation of integrons (Gillings et al. 2009). A correlation between the SOS-response induction and integron integrase-mediated recombination has been elucidated, suggesting that SOS-response induction enhances the exchange of resistance genes under stressful conditions, including antimicrobial treatment (**Fig. 1.3**) (Cambray et al. 2011). Plasmids are genetic elements that harbour QAC and other antimicrobial resistance genes, that promote the effective dissemination of genes in a bacterial population (Elhanafi et al. 2010). An important group of plasmids are the incompatibility (Inc) group, known as IncP-1, these carry extrachromosomal genetic elements that can be transferred to most Gram-negative bacteria (Shintani et al. 2010). These plasmids are commonly distributed in environments where sub-inhibitory levels of QACs are prevalent, such as soil and wastewater settings (Bahl et al. 2009). Suggesting that low-level QAC exposure may drive the continued spread and circulation of resistance genes through HGT within different environments and bacterial populations.

1.7.3 Quaternary ammonium compound linked to antibiotic resistance

The possibility the of co-availability of QAC substances and antibiotics in certain environments raises the concern of whether common modes of action between QACs and some antibiotics can select for biological mutants that can be cross-resistant to both antimicrobial agents.

Additionally, the acquisition of a mobile genetic element could provide some degree of QAC tolerance while the microorganism is being exposed to low concentrations of QACs while facilitating co-selection of antibiotic resistance (Wales and Davies 2015). The uncontrolled and indiscriminate use of QACs can result in the evolution/development of microorganisms having multidrug resistance capabilities through co-resistance mechanisms (Karatzas et al. 2007; Buffet-Bataillon et al. 2012a; Kim et al. 2018). However, none of the clinically important antibiotics shares similarity with the proposed mode of action of QACs (Gilbert and Moore 2005; Davies and Davies 2010).

Generally, antibiotics have more specific cellular target sites as opposed to the proposed broad-spectrum membrane targeting activity of QACs (Davies and Davies 2010). ROS are produced as a result of the antibiotic interactions with their classical targets (Páez et al. 2010; Chittezham Thomas et al. 2013). Similarly, QACs can cause ROS production during the penetration of the cell membrane, including hydrogen peroxide, superoxide anion and hydroxyl radicals (Zhang et al. 2018). Bacteria contain detoxifying proteins able to nullify ROS molecules and counter the damage induced by various pathways, such as *SoxRS*, *OxyRS* and SOS-response regulons (Zhao and Drlica 2014). Although antibiotics and QACs have dissimilar target sites and modes of action, the induction of ROS by administration results in similar regulons being activated and thus similar mechanisms are activated in both instances. The link between the activation of oxidative stress regulons and antimicrobial activity is that certain genes can contribute to broad antimicrobial resistance, such as *acrAb* operon, encoding a multidrug efflux pump (Koutsolioutsou et al. 2001). Multidrug efflux pumps like AcrAB-TolC, have connected antibiotic and QAC resistance (Buffet-Bataillon et al. 2012a). These pumps can decrease susceptibility to ciprofloxacin and fluoroquinolone antibiotics and alkyl dimethyl benzyl ammonium chloride (ADBAC) and didecyl dimethyl ammonium chloride (DDAC) QACs (Buffet-Bataillon et al. 2012a). In addition to multidrug efflux encoding, genes encoding antisense RNA reduced synthesis of outer membrane porins resulting from *SoxRS*

regulon also contribute to the antimicrobial resistance (Koutsolioutsou et al. 2001). Therefore, the mutual selection criteria for resistant populations able to withstand both QAC and antibiotic treatment could be the oxidative stress-induced response. The same oxidative stress response may facilitate the development of cross-resistance between QACs and clinically relevant antibiotics using identical or different mechanisms.

Apart from oxidative stress-induced resistance characteristics, mobile genetic elements play a crucial role in the acquisition of antimicrobial resistance characteristics leading to both antibiotic and QAC resistance (Ciric et al. 2011). These elements can be disseminated between bacteria populations of similar or utterly unique species via HGT, resulting in either co-resistance or cross-resistance to antibiotics and QACs (Sidhu et al. 2002). The conservation of antibiotic and QAC resistance genes on mobile elements is resultant of the selective pressures in natural or industrialised environments providing an advantage to populations harbouring these genes (Russell 2002).

Class 1 integrons harbour diverse *qac* gene cassettes and often the majority of these integrons possess *qac* gene cassettes (Gillings et al. 2009). The widespread distribution of a multitude of *qac* cassettes suggests a common functionality of the efflux pumps encoded by environmental bacteria, as in the case of defence against natural toxins (Gilbert and McBain 2003). Although antibiotic resistance genes are not generally associated with class 1 integrons, the parallel increase of antibiotic and disinfectant resistance together raises the concern that the widespread use of disinfectants selects for antibiotic-resistant bacterial populations (Gilbert and McBain 2003; Bjorland et al. 2005). This co-selection occurs by the acquisition of resistance mechanisms that allow the exclusion of both antibiotics and disinfectants simultaneously, such as efflux pumps or alterations in membrane properties (Langsrud et al. 2004; Poole 2007). Furthermore, the acquisition of a single plasmid/transposon harbouring both antibiotic and disinfectant resistance mechanisms co-

select for dual resistance capabilities (White and McDermott 2001; Gaze et al. 2005). All novel acquired mobile genetic elements had some origin from natural sources. However, their transfer from the natural environment into key human-associated environments must have been driven by the selection of newly acquired phenotypes adapted to withstand increasingly harmful environments because of the negligent use of antimicrobials.

1.8 Efflux pumps

Efflux is the removal/pumping out of a solute from within the cell (Piddock 2006). Efflux pumps reduce the accumulation of toxic compounds within the cell, providing the bacterium opportunity to adapt to the antimicrobial/stressor through mutational events allowing a greater possibility for survival (Li and Nikaido 2009). Genes that encode cell membrane proteins or regulatory proteins of efflux pumps aid in the antimicrobial challenge, helping to maintain the survival of bacteria (Li et al. 2015). Efflux pumps may have substrate specificity for a single compound or may be able to transport a broad range of unrelated compounds and thus are classified as MDR transporters (Piddock 2006). MDR transporters can provide resistance toward relevant antibiotics and QACs (Buffet-Bataillon et al. 2012b). The energy-dependent transportation of compounds by efflux pumps occurs without altering the structure of the compound (Kumar and Schweizer 2005).

Efflux pumps can be divided into two major classes containing five multidrug efflux superfamilies based on bioenergetic requirements and structural criteria. The first class is the primary active transporters consisting of the adenosine triphosphate (ATP)-binding cassette (ABC)-type transporters that utilise the energy of ATP hydrolysis to facilitate transport of substrates (Putman et al. 2000) (**Fig. 1.4**). The second class containing the remaining four superfamilies is the secondary active transporters that utilise the transmembrane electrochemical proton/sodium ion gradients to facilitate transport of substrates (Putman et al. 2000). The remaining four superfamilies are the drug/metabolite transporter (DMT)

superfamily containing the significant small multidrug resistance (SMR) subfamily, major facilitator superfamily (MFS), multidrug/oligosaccharidyl-lipid/polysaccharide flippase (MOP) superfamily containing the significant multi-antimicrobial extrusion (MATE) subfamily and the RND superfamily (Mahamoud et al. 2007) (**Fig. 1.4**). Recently, a novel family of efflux pumps have been elucidated in *Acinetobacter baumannii* known as the proteobacterial antimicrobial compound efflux (PACE) family (Hassan et al. 2015) (**Fig. 1.4**).

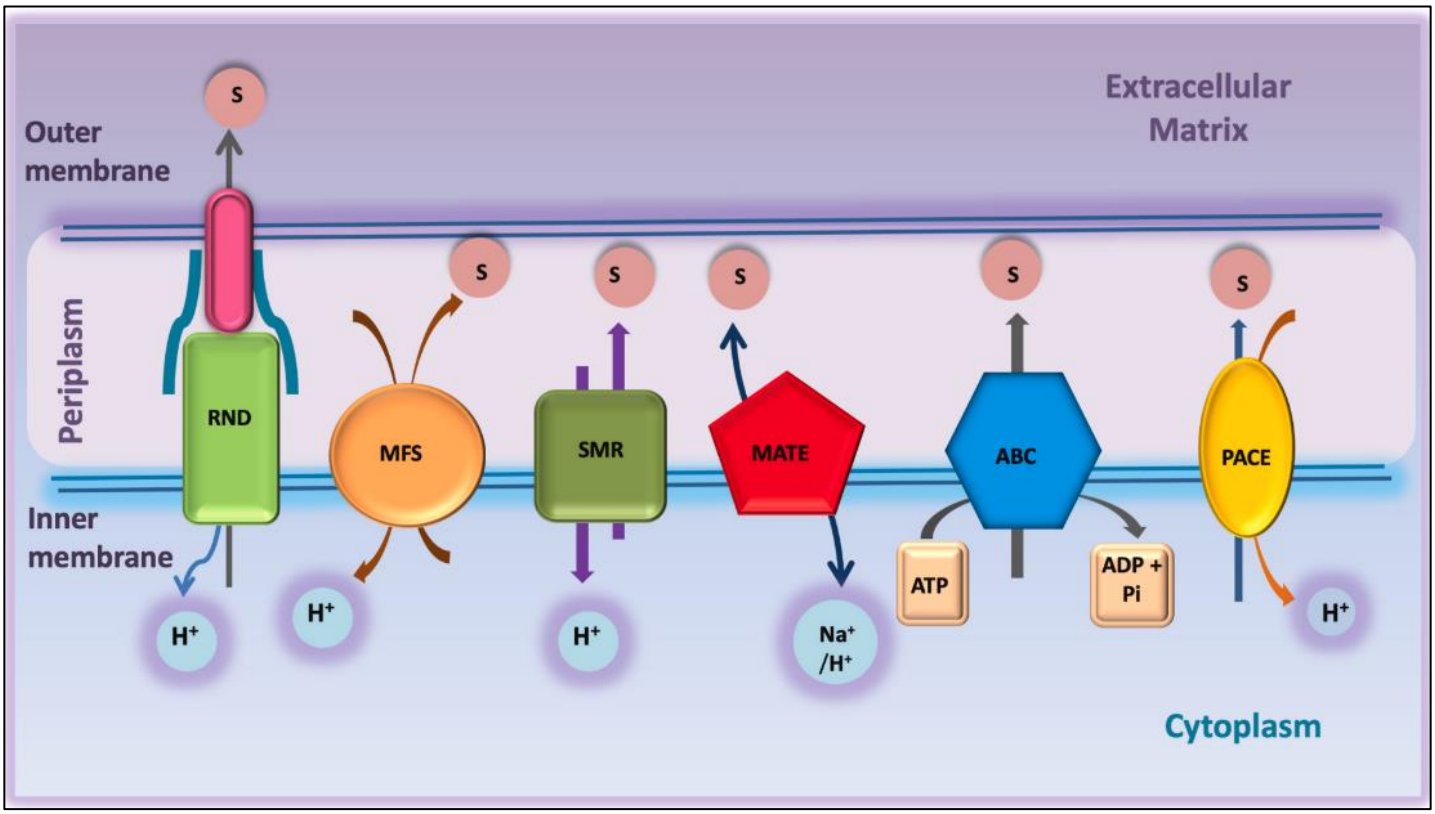

Figure 1.4: A diagrammatic representation of the five main superfamilies of efflux pumps, including three families. The RND, MFS, ABC, MOP, and DMT are the superfamilies. The SMR subfamily forms part of the DMT superfamily and the MATE subfamily forms part of the MOP superfamily. The PACE family is a newly characterised (Verma et al. 2021).

1.8.1 ATP-binding cassette superfamily

This superfamily can utilise ATP to transport a variety of substances, such as sugars, amino acids, ions, drugs, polysaccharides and proteins (Wilkens 2015). ABC transporters can form multi-protein complexes incorporating integral membrane proteins and ATPase-functioning cytoplasmic membrane proteins (Kumar and Schweizer 2005). The minimal structural organisation of an ABC transporter includes four domains, two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs) (Higgins et al. 1986). Six transmembrane α-helices in either homo or heterodimers form the TMD segments (Dawson and Locher 2006). The NBDs bind the ATP molecules on the cytoplasmic membrane side functioning in conjunction with the TMDs (Lubelski et al. 2007). The binding of ATP results in conformational changes in the transporter structure, allowing the export/import of substrates (Zgurskaya and Nikaido 2000).

1.8.2 Drug/metabolite transporter superfamily

The DMT superfamily has a clinically relevant subfamily in the SMR transporters (Jack et al. 2001). These proteins consist of approximately 110 amino acids with four transmembrane segments and are energised by the PMF (Kumar and Schweizer 2005). These proteins have been shown to export various classes of antibiotics, disinfectants, dyes, and detergents (Srinivasan et al. 2009; Srinivasan and Rajamohan 2013). Some of the better-investigated pumps of the SMR subfamily are the Smr protein in *S. aureus* and EmrE protein in *E. coli* (Yerushalmi et al. 1995; Bjorland et al. 2001). Furthermore, clinically relevant Qac proteins form part of the SMR subfamily. These *qac* genes are widespread among clinical and environmental bacteria but are particularly linked to staphylococci and enterococci in Gram-positive bacteria (Bischoff et al. 2012; Akin et al. 2020). These genes are also present in Gram-negative bacteria, such as *Pseudomonas*, *Acinetobacter*, *Aeromonas* and *Enterobacteriaceae* sp. (Kücken et al. 2000; Chang et al. 2007; Mak et al. 2009). The prevalence of *qac* genes in

Gram-negative bacteria is related to the high occurrence of class 1 integrons (Gaze et al. 2005).

1.8.3 Major facilitator superfamily

Members of the MFS can function as uniport, symport or antiport systems able to transport metabolites, sugars, anions or drugs (Kumar and Schweizer 2005). Generally, these proteins function as single-component transporters, such as NorA, but can form complexes with a membrane fusion protein (MFP) and outer membrane protein (OMP) components, such as EmrAB-TolC (Yoshida et al. 1990; Lomovskaya and Lewis 1992). These membrane-associated proteins usually are comprised of 12- or 14-transmembrane segments (Pao et al. 1998). The MFS contains three subfamilies involved in drug efflux, drug/H^+ antiporter (DHA)1, DHA2 and DHA3 (Kumar and Schweizer 2005). The DHA1 and DHA2 subfamilies are known to have very broad substrate specificity for structurally distinct molecules, whereas the DHA3 subfamily is known to have greater substrate specificity for antibiotics (Kumar and Schweizer 2005).

1.8.4 Multidrug/oligosaccharidyl-lipid/polysaccharide flippase superfamily

The MOP superfamily has a clinically relevant subfamily known as the MATE subfamily. These MATE efflux pumps have been identified in various bacterial species, such as *Vibrio parahaemolyticus* (NorM; VmrA), *Vibrio cholerae* (VcrM; VcmA), *P. aeruginosa* (PmpM) and *E. coli* (YdhE) (Morita et al. 2000; Huda et al. 2001, 2003; He et al. 2004). Unique to the MATE transporters is the ability to utilise two sources of energy for transport, the PMF and the sodium ion gradient (Morita et al. 1998; He et al. 2004).

1.8.5 Resistance-nodulation-cell division superfamily

RND transporters are generally chromosomally encoded but certain plasmid-encoded RND proteins have been identified (Hansen et al. 2004). RND efflux systems function using substrate/H^+ antiport mechanisms, exchanging the substrate for the hydrogen ion in opposite directions of the cell membrane (Kumar and Schweizer 2005). These efflux pumps are well-known to contribute to either intrinsic or acquired resistance to a range of antimicrobials in Gram-negative bacteria (Blair and Piddock 2009). Generally, in Gram-negative bacteria, these pumps form tripartite complexes consisting of the 12-transmembrane segments (Verma et al. 2021). The RND membrane protein component is generally associated with an MFP and OMP, to facilitate transport across both membranes (Kumar and Schweizer 2005). However, in Gram-positive bacteria, these pumps lack association with MFP and OMP components (Paulsen et al. 1996). Some of the best-characterised members of this superfamily are AcrAB-TolC of *E. coli*, AdeABC of *A. baumannii* and MexAB-OprM of *P. aeruginosa* which can efflux a very broad range of substrates. These substrates include antibiotics, heavy metals, detergents, disinfectants and many other unrelated compounds (Poole et al. 1993; Ma et al. 1995; Poole 2002). *S. marcescens* a notorious nosocomial pathogen also has RND transporters, SdeAB and SdeXY, that contribute to resistance against multiple antibiotics (Chen et al. 2003b; Maseda et al. 2009; Iguchi et al. 2014).

1.9 Efflux pump inhibitors

The clinical significance of efflux pumps in providing antimicrobial resistance to both Gram-positive and Gram-negative bacteria provides a unique opportunity to develop countermeasures. The development of combination therapies against the activity of specifically multidrug membrane transport proteins as drug targets is an attractive approach (Lomovskaya and Watkins 2001). Efflux pumps are attractive targets for combination therapy approaches using synthetic or plant-derived efflux pump inhibitors (EPIs) as a possible means

to curb the spread of multidrug resistance by assisting antibiotic treatments of stubborn bacterial infections (Opperman and Nguyen 2015; Alibert et al. 2017). Specific targeting of the RND transporter is vital since these efflux pumps are continuously involved in the increased levels of MDR in clinical bacteria (Lomovskaya and Bostian 2006).

The inhibition of efflux pumps can be achieved in numerous ways:

i) inhibiting the binding of the substrate through competitive or non-competitive inhibition with a non-antibiotic molecule (**Fig. 1.5**).

ii) inhibiting the interactions between multi-component efflux systems that are required for adequate functional assembly (**Fig. 1.5**).

iii) inhibiting the supply/availability of energy sources of the efflux pump (**Fig. 1.5**).

iv) inhibiting the regulatory pathways responsible for the expression or repression of efflux pumps (**Fig. 1.5**).

v) blocking the outer membrane channel (**Fig. 1.5**).

vi) altering the current chemical structure of commonly used antibiotics to reduce the binding affinity of efflux substrate recognition sites (**Fig. 1.5**) (Pagès and Amaral 2009).

These inhibition avenues will promote the successful accumulation of antimicrobial drugs inside the bacterial cell to enhance the antimicrobial effect (Pagès and Amaral 2009). Thus, EPIs can become promising tools to decrease intrinsic resistance toward antibiotics, reducing the impact that acquired resistance can confer and potentially curtail the emergence of resistant mutants (Agreles et al. 2021). Although the possible ways to exploit efflux pumps have been deciphered, the practical application of these methods has not yet been implemented in the commercial environment (Sharma et al. 2019).

For a chemical agent to be successfully implemented as an EPI, it would need to undergo stringent testing and meet specific requirements (Van Bambeke et al. 2006). The molecule must lack intrinsic antibacterial properties as this would promote the selection of resistant mutants thereby severely restricting the utility of the molecule (Van Bambeke et al. 2006). The molecule must also be selective for bacterial efflux pumps, not targeting eukaryotic efflux pumps. the molecule must have ideal pharmacological characteristics, such as high therapeutic and safety indices and good absorption, distribution, metabolism, excretion, and toxicity profile. Finally, the molecule must be economically feasible for production at a commercial level (Van Bambeke et al. 2006).

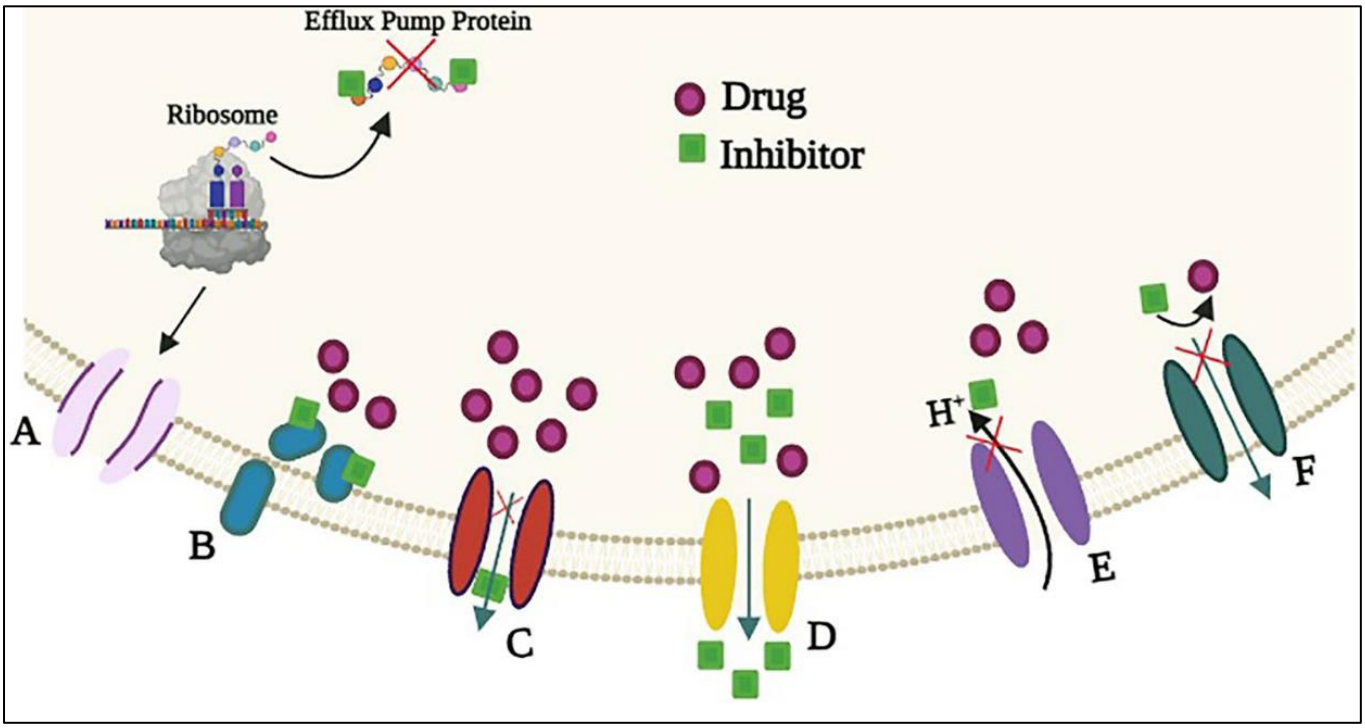

Figure 1.5: Efflux inhibitory molecules can induce functional inhibition by (A) changing the expression regulation pathways of efflux pumps; (B) hindering the functional assembly of efflux pumps into complete structural systems; (C) blocking the membrane channel to prevent actual transport of substrates after binding; (D) by competitive or non-competitive activity at substrate binding sites or other sites vital for functional transportation; (E) collapsing the

energy gradient required for efflux; (F) modifying the drug to prevent efflux (Agreles et al. 2021).

1.9.1 Energy dissipation

This form of efflux inhibition does not require the interaction of the inhibitory molecule and the efflux pump (**Fig. 1.5** (E)). However, all efflux pumps require cellular energy for functioning, either provided by the PMF or ATP hydrolysis (Putman et al. 2000). When considering the most clinically relevant efflux pumps conferring multidrug resistance, majority of these classes of transporters are categorised as efflux pumps that require the PMF for energy (Piddock 2006). Thus, targeting this energy source for disruption would be advantageous to eliminate a multitude of efflux determinants.

A well-known energy dissipator is carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which is classified as an ionophore because it disrupts the PMF by influencing both of the parameters that compose the PMF: the electric potential ($\Delta\psi$) and the transmembrane proton gradient (ΔpH) (Farha et al. 2013; Park and Ko 2015). This compound also affects the metabolic state of bacterial cells making them inactive, initiating the debate about whether the synergistic effect of CCCP with antibiotics is because of efflux pump inhibition or metabolic inactivation of the cell. The ambiguous nature of the inhibitory mechanism of CCCP and its cellular toxicity toward mammalian cells has restricted its introduction into commercial settings (Sharma et al. 2019). CCCP has been used to improve the effectiveness of various classes of antibiotics in numerous clinically relevant bacteria, such as *A. baumannii*, *Salmonella enterica* and *Klebsiella* sp. (Braoudaki and Hilton 2005; Fenosa et al. 2009; Sanchez-Carbonel et al. 2021). CCCP has been also shown to reverse the insusceptibility of efflux pumps to various disinfectants, such as BAC, triclosan and cetylpyridinium chloride (Nagai et al. 2003; Braoudaki and Hilton 2005).

1.9.2 Competitive or non-competitive binding

This form of efflux inhibition requires direct interaction between the inhibitory molecule and the efflux pump (**Fig. 1.5** (D)). A well-known inhibitor is the plant alkaloid, reserpine (RSP), which binds directly to specific amino acid residues of the efflux pump protein to inhibit functional activity (Stavri et al. 2007; Verma et al. 2021). RSP has been shown to effectively inhibit the efflux of tetracycline in *Bacillus subtilis* by binding to the Bmr protein recognising specific amino acids (Neyfakh et al. 1991). This EPI has also been shown to potentiate the activity of tetracycline in clinically significant isolates of MRSA possessing Tet(K) proteins (Gibbons and Udo 2000). Furthermore, RSP was able to reverse NorA-conferred multidrug resistance and was able to enhance the antibacterial activity of norfloxacin and fluoroquinolones in *S. aureus* (Neyfakh et al. 1993; Kaatz and Seo 1995).

The *in vitro* inhibitory activity of RSP against efflux pumps is well documented, however, RSP is also used as an antihypertensive drug in clinical practice limiting its use due to inadequate pharmacokinetics and toxicity (Buffet-Bataillon et al. 2012b). RSP specifically targets the MFS, ABC and RND efflux superfamilies (Stavri et al. 2007; Garvey and Piddock 2008). RSP can recognise and bind specific amino acid residues in the protein structure (Stavri et al. 2007; Verma et al. 2021). Concerningly, RSP has demonstrated the ability to select multidrug-resistant *Streptococcus pneumoniae* strains (Garvey and Piddock 2008). ABC transports PatA and PatB confer multidrug resistance in *S. pneumoniae*, but only PatA has substrate specificity for RSP (Garvey and Piddock 2008). The amino acids to which RSP can bind are unknown; however, if these residues are corresponding to those used during the transportation of antimicrobial agents' joint resistance could occur. This joint specificity could relate the non-competitive nature of RSP inhibition to that of competitive. The danger especially for competitive inhibition is that if the EPI and the combined antimicrobial agent are substrates for the same efflux pump, common use may drive resistance development of both compounds and further unintended cross-resistance (Garvey and Piddock 2008). To circumvent this

diagnostic assay testing should be utilised to differentiate between antimicrobial resistance traits and traits that affect EPI activity.

1.10 Liquid chromatography-mass spectrometry

Liquid chromatography-mass spectrometry (LC-MS) is an analytical measuring technique that combines the technological aspects of liquid chromatography (LC) and mass spectrometry (MS) (Agilent Technologies 2001). The LC segment of the technique provides the required resolving power and the MS allows detection specificity of the molecule being investigated (Agilent Technologies 2001). LC separates the sample into various fragmented components depending on elution from the chromatographic solid phase and then the mass spectrometer detects the charged ions of the fragmented sample (Agilent Technologies 2001). The mass spectrophotometer converts the eluted analyte components into a charged/ionised state. The fragment ions produced during the ionisation process are based on the mass-to-charge ratio (*m/z*) of the elute component (Pitt 2009). The combination of these techniques can generate information about the molecular weight, structure, identity and quantity of specific components of the sample previously unattainable using gas chromatography/mass spectrometry (Agilent Technologies 2001). The conjunction of LC with MS significantly expanded the analytical capacity of MS to include a greater range of organic compounds from small pharmaceutical agents to large proteins (Agilent Technologies 2001).

Cationic surfactants, such as QACs, have been investigated using various types of analytical combinations. These include thin-layer chromatography coupled with flame ionisation, liquid chromatography, gas chromatography, nuclear magnetic resonance and capillary electrophoresis (Martínez-Carballo et al. 2007). The liquid chromatography methods alone are related to conductivity and indirect UV detection of samples has disadvantageous aspects as they lack analytical specificity (Martínez-Carballo et al. 2007). However, LC-MS is a technique

that can be used for environmental sample analysis. Specifically, an analysis of QACs has been conducted since there is a greater sensitivity of LC-MS compared to other liquid chromatography techniques (Castro et al. 1999; Ford et al. 2002; Martínez Vidal et al. 2004).

Although the addition of mass spectrometry allows for better detection and thus the problem to be overcome, an enrichment step is required before chromatographic analysis. The enrichment will allow the determination of low limits of detection of QAC substances (Martínez-Carballo et al. 2007). Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are techniques used with the filtration of water samples before extraction allowing for sample clean-up and pre-concentration before analysis (Radke et al. 1999; Castro et al. 2000). SPE involves the separation of solutes between two phases, one liquid and the other a solid phase (Żwir-Ferenc and Biziuk 2006). SPE is a favourable technique since it is economical and simple to perform (Castro et al. 2000). This technique is also advantageous because of its versatility and applicability for environmental sample analysis, often being recommended for isolation and concentration of QACs (Picó et al. 2000). The basic principle encompasses the passing of a liquid sample through the column containing the two phases with an absorbent property which retains passing analytes. Once the entire sample has been administered to the column and passed through the sorbent, analytes that are attracted and retained will be recovered after elution with adequate solvent (Camel 2003).

Being able to quantify the presence of contaminants in drinking water is an important analytical tool. Thus, the ability to quantify QACs in herbicides is important to quantify and regulate the levels of disinfectant contamination (Castro et al. 2000, 2001). The use of LC-MS as a detection method cannot detect contaminants within the low limit of detection as defined by the European Community of 100 ng/l for pesticides, therefore a trace enrichment step is required before chromatographic analysis (Castro et al. 2000). Successful detection of QACs in marine environments have been conducted using LC-MS (Bassarab et al. 2011).

Furthermore, metabolites of pesticides have also been detected in the environment, suggesting that LC-MS is an acceptable technique for analysing QAC spiked environmental samples because of more sensitive detection parameters compared to other liquid chromatography detection methods (Bassarab et al. 2011).

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Chapter 2: Susceptibility tests and predictions of transporter profile in *Serratia* species

2.1 Introduction

Serratia marcescens is recognised as an important nosocomial pathogen able to cause urinary tract, bloodstream, and many other medical-associated infections (Yu 1979; Körner et al. 1994; Su et al. 2003). *S. marcescens* has been suggested to have intrinsic and acquired resistance toward antimicrobial agents (Mahlen 2011). Specifically focusing on disinfectants, such as chlorinated handwash, have been in use since the 19th century (Rotter 2001). However, the advent of cationic biocides, such as biguanides, quaternary ammonium compounds (QACs), phenolics and aldehydes, in the 20th century catapulted the use of newly discovered disinfection agents (Maillard 2005). Disinfectants have become an important part of biosecurity treatments in veterinary settings (Bragg and Plumstead 2003). However, with the rising threat of antibiotic-resistant and multidrug-resistant bacteria in clinical settings, disinfectant and antibiotic therapies must be joined together to combat this major health concern worldwide (Roca et al. 2015).

One of the best-studied modes of disinfectant resistance and multidrug resistance (MDR) is efflux pumps. QAC efflux is generally mediated by *qac* resistance genes that code for proteins that are part of two secondary transporters (Tennent et al. 1985; Bjorland et al. 2003). MDR is generally mediated by multidrug resistance-nodulation-division (RND) superfamily proteins (Blair and Piddock 2009). QAC-specific genes, such as *qac, mde* and *smr* genes have been identified on different plasmids, while, other genes of different transporter families can be chromosomally encoded (Bjorland et al. 2003; Huang et al. 2004).

This chapter aimed to investigate which efflux pumps in two *Serratia* strains, namely *S. marcescens* subsp. *marcescens* ATCC 13880 and *Serratia* sp. HRI could provide QAC and

multidrug resistance. To achieve this the efflux pump repertoire of these strains was investigated by an annotation pipeline predicting the identity and function of each protein. Furthermore, susceptibility testing was conducted on the strains using specific disinfectants to establish the minimum inhibitory concentration (MIC) thresholds. The extent of efflux pump activity was determined by the addition of two distinct efflux pump inhibitors (EPIs) *in vitro*. This chapter is presented as a manuscript (Susceptibility Tests and Predictions of Transporter Profile in *Serratia* Species) that has been published in the peer-reviewed, accredited journal "**Microorganisms"**. As the first author, I researched the topic and completed the investigation of the efflux pump profile predictions and the susceptibility tests comparing the two *Serratia* strains. I wrote and compiled the article in conjunction with the co-authors of this article. The authors of this article were Ms Samantha J. Mc Carlie, my two supervisors on my M.Sc. project, Prof. Robert R. Bragg, and Dr Charlotte E. Boucher-van Jaarsveld, who were integral to the initial conceptualisation, review and editing of the paper. This published article is presented as the complete Chapter 2 of this thesis.

2.2 Published manuscript

microorganisms

MDPI

Article

Susceptibility Tests and Predictions of Transporter Profile in *Serratia* Species

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Abstract: Disinfectants and biosecurity are critically important to control microbial diseases. Resistance to disinfectants compromises sectors such as agriculture and healthcare systems. Currently, efflux pumps are the most common mechanism of antimicrobial resistance. This study aimed to identify the efflux transporters responsible for disinfectant resistance in a multidrug-resistant isolate *Serratia* sp. HRI compared to a susceptible *Serratia* sp. type strain. An efflux system profile was generated using the Transporter Automatic Annotation Pipeline (TransAAP) for both isolates. Thereafter, the efflux pump inhibitors, reserpine (RSP) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used to reveal the role of efflux pumps in susceptibility to three disinfectants (Didecyldimethylammonium chloride, HyperCide®, and benzalkonium chloride). Interestingly, the resistant isolate had fewer efflux systems in total compared to the type strain and fewer efflux systems classified as resistance efflux pumps. After the addition of RSP, a significant reduction in resistance capabilities against all three antimicrobials was observed for both isolates. However, CCCP supplementation produced mixed results with some outcomes suggesting the involvement of the Eagle effect. This study provides evidence that efflux pumps are responsible for the disinfectant resistance phenotype of the *Serratia* species due to the increased susceptibility when efflux pump inhibitors are added.

Keywords: disinfectant resistance; efflux pump; reserpine; carbonyl cyanide 3-chlorophenylhydrazone; *Serratia marcescens*; multidrug-resistance

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1. Introduction

The *Serratia* genus is categorized as part of the *Enterobacteriaceae* family, and members are commonly found in soil and water associated with plants, insects, animals, and humans [1]. *Serratia marcescens* is most frequently associated with human infections [2]. Clinical isolates harbor intrinsic chromosomal and acquired plasmid-encoded elements conferring resistance toward many antibiotics and disinfectants [1].

Incorporating disinfectants as a preventative measure, in combination with antibiotic administration, has become an integral part of infection control. However, the rising threat of antibiotic and multidrug resistance accompanied by opportunistic pathogens is a worldwide health concern [3]. This rise of untreatable/resilient bacterial populations increases the need for effective sterilization/disinfection methods that prevent the proliferation and spread of resistance [4]. Resistance against quaternary ammonium compounds (QACs) has been reported in clinical, industrial, and veterinary environments, and resistance genes specific to QACs have been identified [5]. One of the best-studied modes of disinfectant resistance is efflux systems in the small multidrug resistance (SMR) subfamily and major facilitator superfamily (MFS) [6,7]. These systems consist of cytoplasmic membrane-embedded multidrug transporters that recognize and expel toxic compounds inside the cell to the external environment [8].

In this study, the genomes of *S. marcescens* subspecies *marcescens* strain ATCC 13880 and multidrug-resistant isolate *Serratia* species Highly Resistant Isolate (HRI) [9] were submitted

to a prediction pipeline tool to compare efflux system profiles and identify any resistance efflux systems [10]. Susceptibility testing was conducted using three test disinfectants against each *Serratia* isolate and two efflux pump inhibitors (EPIs) were added to determine efflux pump activity. Two standard EPIs were chosen, reserpine (RSP) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP). RSP inhibits efflux by binding specific amino acid residues on the efflux pump, causing inhibition in certain efflux families, including the MFS, resistance-nodulation-division (RND) superfamily, and ATP-binding cassette (ABC) superfamily [11,12]. CCCP is classified as a protonophore as it disrupts the proton motive force (PMF) requirements of efflux pumps of the secondary transporter classification [13,14]. This inhibitor affects efflux systems belonging to the MFS, RND, drug/metabolite transporter (DMT), and multidrug/oligosaccharidyl-lipid/polysaccharide flippase (MOP) superfamilies [13,14].

This study aimed to first generate a list of predicted transporters in each *Serratia* isolate. Secondly, from the predicted transporters, individual transporters indicating resistance function were grouped, specifically grouping functionality/substrate specificity for QAC and multidrug resistance. Thirdly, the susceptibility/resistance profiles of the *Serratia* isolates were determined using test disinfectants. Finally, this work revealed a possible link between the resistance expressed and transporters present in the genomes.

2. Materials and Methods

2.1. Bacterial Strains

S. sp. HRI was isolated in 2018 from a DDAC-based disinfectant bottle (1% *v/v*) at room temperature at the University of the Free State in South Africa [9]. *S.* sp. HRI can be accessed on NCBI as BioProject PRJNA580358, BioSample SAMN13155787, and the DDBJ/ENA/GenBank database under accession number WIXF00000000. *S. marcescens* subsp. *marcescens* ATCC 13880 strain was obtained from the American Type Culture Collection in 2018 for use at the Veterinary Biotechnology Laboratory at the University of the Free State. *S. marcescens* subsp. *marcescens* ATCC 13880 can be accessed on NCBI as BioProject PRJNA716961, BioSample SAMN18473280, and the GenBank database under accession number CP072199.1. Bacterial isolates were obtained from the University of the Free State's bacterial culture collection. The isolates were aerobically grown at 37 °C for 18–20 h in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA) plates for routine bacterial cultivation.

2.2. Preparation of Disinfectants and Inhibitors

Benzalkonium chloride (BAC) (Sigma-Aldrich, St. Louis, MI, USA), didecyldimethylammonium chloride (DDAC), and HyperCide® (14% peracetic acid (CH_3CO_3H) and 22% hydrogen peroxide (H_2O_2)) (ICA International Chemicals, Stellenbosch, ZAF) were used in this study. The disinfectants were freshly prepared in sterile distilled H_2O before each experimental requirement. RSP (Sigma-Aldrich, St. Louis, MI, USA) and CCCP (Sigma-Aldrich, St. Louis, MI, USA) were used at working concentrations of 5 µg/mL and 12.5 µM, respectively.

2.3. Efflux Pump Predictions and Annotations

The whole genome sequences of *S.* sp. HRI and *S. marcescens* subsp. *marcescens* ATCC 13880 were obtained from the National Centre of Biotechnology Information (NCBI) and are accessible using the following identification codes: *S.* sp. HRI: [GenBank]: 29000988, [Taxonomy I.D]: 2663241 and *S. marcescens* subsp. *marcescens* ATCC 13880: [GenBank]: 25904888, [Taxonomy I.D]: 911022. The transporter profile of both isolates was assessed using the Transporter Automatic Annotation Pipeline (TransAAP) tool (http://www.membranetransport.org/transportDB2/index.html (accessed on 16 August 2022)), developed by Professor Ian Paulsen, Dr. Liam Elbourne, Dr. Karl Hassan and Dr. Sasha Tetu, 2017 [15]. The TransAAP tool utilizes a relational database to predict the cytoplasmic membrane protein complement of organisms whose whole genome sequences are available by inserting the taxonomic I.D. The transporter proteins of each organism were classified into protein families according to the transporter classification system, and substrate/function

predictions were provided for each transporter protein. Each entry was individually curated to identify resistance functionality, such as antibiotic, disinfectant, multidrug or heavy-metal resistance. The identified resistance transporters were further verified using the NCBI BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 17 August 2022), UniProt BLAST (https://www.uniprot.org/blast/ (accessed on 17 August 2022)), and Kyoto Encyclopedia of Genes and Genomes (KEGG) BLAST (https://www.genome.jp/tools/blast/ (accessed on 17 August 2022)). The transporter identity was deemed accurate depending on the findings of the three BLAST results.

2.4. Susceptibility Testing

The disinfectant resistance profiles of *S. marcescens* subsp. *marcescens* ATCC 13880 and *S.* sp. HRI, were determined by minimum inhibitory concentrations (MICs) using the broth microdilution method so that the HRI isolate could be classified as resistant or susceptible compared to the ATCC strain. The disinfectants were chosen based on categorical requirements such as a first-generation QAC, a fourth-generation QAC, and a non-QAC. One to two colonies were picked and resuspended in PBS. These suspensions were adjusted to a 0.5 McFarland standard (around 10^8 CFU/mL). A two-fold dilution range was prepared by serially diluting a stock concentration of disinfectant. Then, the bacterial suspensions were added to each diluted disinfectant solution in the range for the contact time. After the contact time of 20 min, each dilution was transferred to brain heart infusion (BHI) broth and incubated overnight. The MICs were determined as the lowest concentration that prevented visible growth. All MICs included at least three technical replicates and three biological replicates.

2.5. Efflux Pump Inhibitor-Supplemented Susceptibility Tests

The efflux pump activity of the *Serratia* isolates was assessed using RSP (Sigma-Aldrich, St. Louis, MI, USA) at a final concentration of 5 µg/mL and CCCP (Sigma-Aldrich, St. Louis, MI, USA) at a final concentration of 12.5 µM. These EPIs were added by using a broth microdilution method. One to two colonies of each strain were picked and resuspended in PBS. The suspensions were adjusted to a 0.5 McFarland standard. A two-fold dilution range was prepared by serially diluting a stock concentration of test disinfectant. Then, the EPIs were added to each dilution to make up the final concentrations, followed by bacterial suspensions to each serial disinfectant dilution. After the 20 min contact time expired, each dilution was transferred to BHI broth and incubated overnight. The EPI control consisted of the final concentration of each EPI and bacterial suspensions to ensure no intrinsic antibacterial activity at concentrations used in experiments. The MIC was determined as the lowest disinfectant concentration under the test that prevented visible bacterial growth in microcentrifuge tubes after incubation. All MIC experiments included at least three technical replicates and three biological replicates.

2.6. Statistical Analysis

The data presented in Figures 1 and 2 depicts the frequency of various types of efflux pump superfamilies produced by a prediction pipeline. These data were analyzed using the chi-squared test on SAS 9.4. The data presented as bar graphs (Figures 3–5) depicts the mean disinfectant concentrations ± standard deviation (n = 3 biological replicates) and was analyzed using SAS 9.4. Data from Figure 3 were compared using two-sample t-tests assuming equal/unequal variances based upon the f-test for the variance of two groups. $p < 0.05$ was considered statistically significant. Data from Figures 4 and 5 were compared using a multiple comparison of samples using the Bonferroni-adjusted ANOVA. $p < 0.05$ was considered statistically significant.

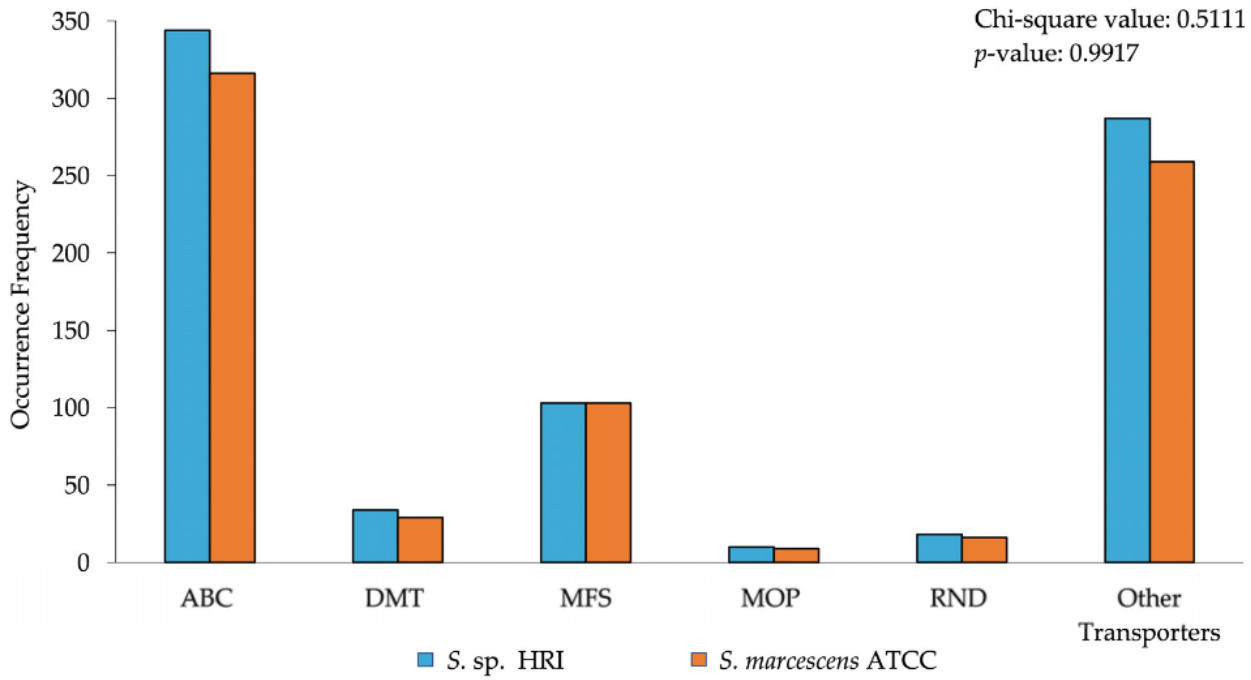

Figure 1. Total transporter predictions of *Serratia* isolates into five main superfamilies of efflux pumps. The *p*-value of the chi-square test > 0.05; therefore, the *Serratia* isolates, and occurrence frequency of efflux pump superfamilies are independent.

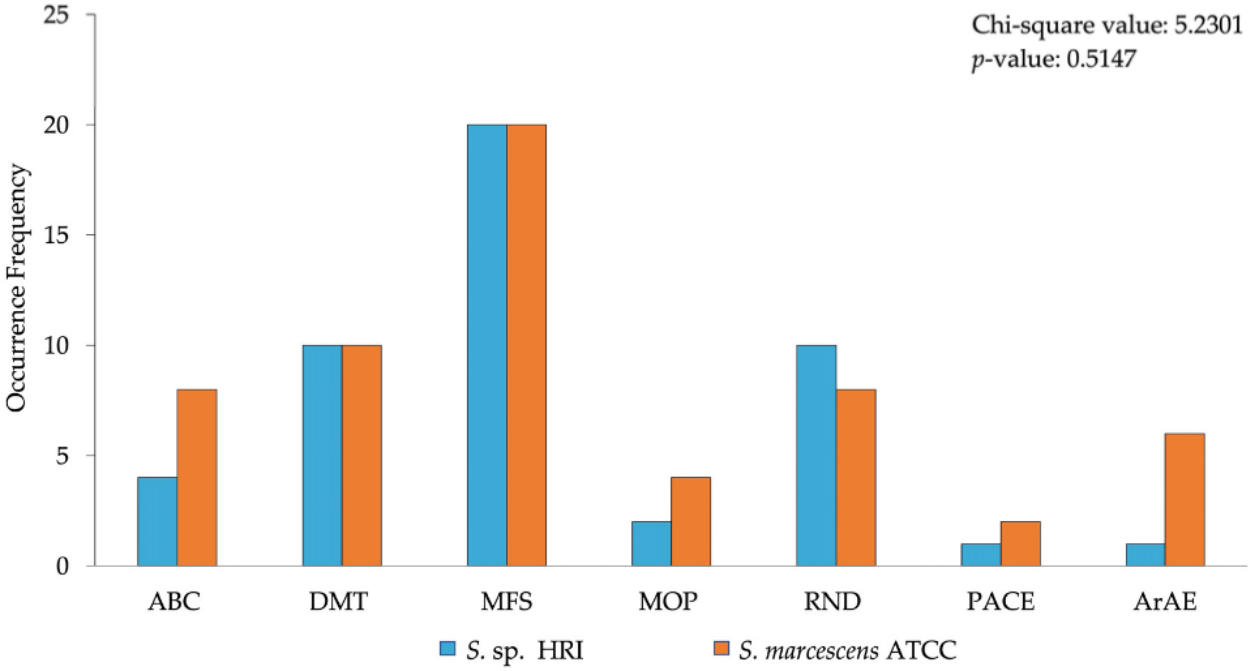

Figure 2. Resistance transporter predictions of *Serratia* isolates into various efflux families. The *p*-value of the chi-square test > 0.05; therefore, the *Serratia* isolates, and occurrence frequency of efflux pump families are independent.

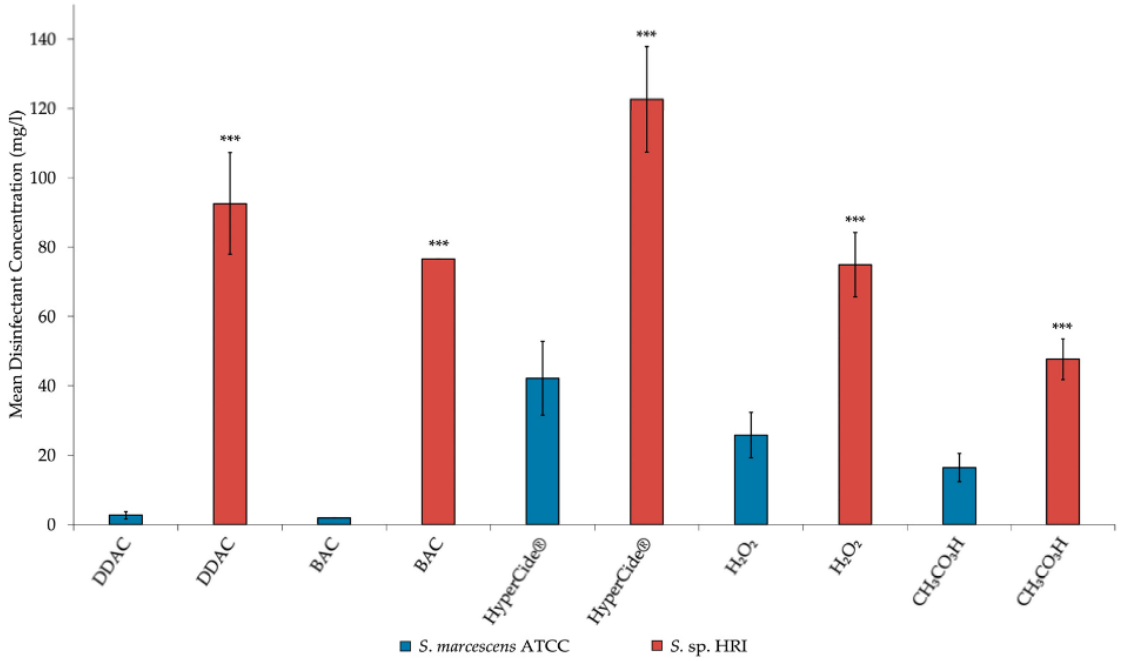

Figure 3. Mean disinfectant concentration of the MIC of *Serratia* isolates. Bars: geometric mean ± 95% C.I. as error bars (n = 3 biological replicates). Significance of difference to type strain indicated by asterisks: *** $p < 0.001$ (two-tailed unpaired t-test of disinfectant concentrations).

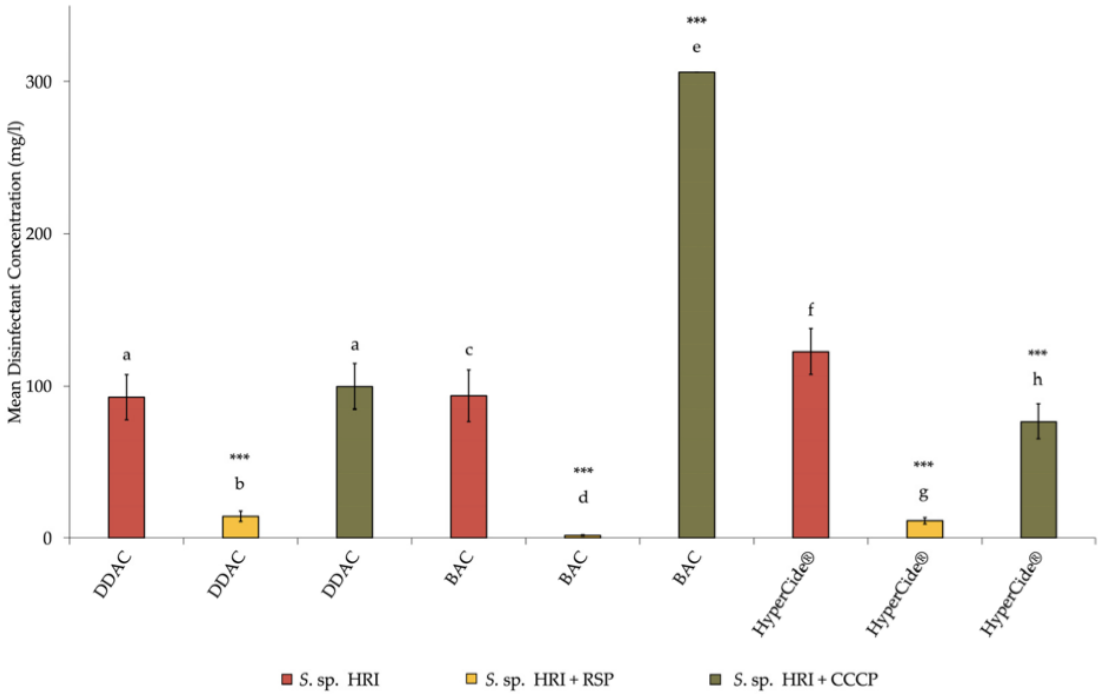

Figure 4. Mean disinfectant concentration of MIC for *S.* sp. HRI with supplementation of EPIs RSP at 5 µg/mL and CCCP at 12.5 µM final concentrations. Bars: geometric mean ± 95% C.I. as error bars (n = 3 biological replicates). Significance of difference to HRI isolate with only disinfectant indicated by asterisks: *** $p < 0.001$ (multiple comparison Bonferroni-adjusted ANOVA). Different letters indicate differences to HRI isolate with only disinfectant.

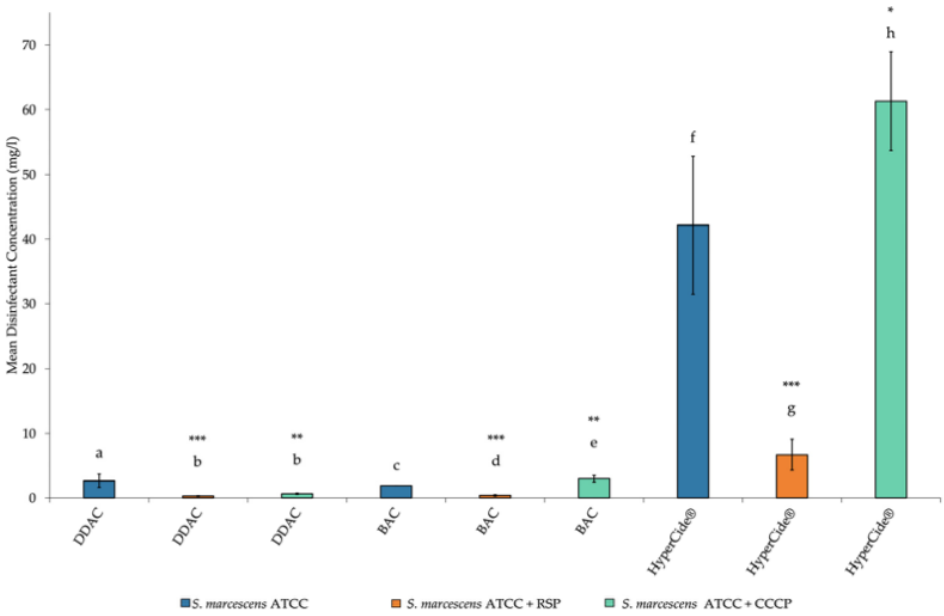

Figure 5. Mean disinfectant concentration of MIC for *S. marcescens* subsp. *marcescens* ATCC 13880 with supplementation of EPIs RSP at 5 μg/mL and CCCP at 12.5 μM final concentrations. Bars: geometric mean ± 95% C.I. as error bars (n = 3 biological replicates). Significance of difference to ATCC strain with only disinfectant indicated by asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (multiple comparison Bonferroni-adjusted ANOVA). Different letters indicate differences to ATCC isolate with only disinfectant.

3. Results

3.1. Efflux System Profile of Isolates

Efflux pump gene arrangements and efflux pump genes associated with antimicrobial resistance were identified in both *Serratia* isolates. Both isolates had representative transporters from all the efflux pump superfamilies, although the relative abundance of specific transporters varied (Figure 1). The efflux pump profile of *S. marcescens* subsp. *marcescens* ATCC 13880 displayed most of the identified transporters were from the ABC superfamily followed by the MFS family (Figure 1). The DMT RND and MOP superfamily transporters constituted a tiny portion of the total transporter repertoire (Figure 1). Finally, the greater portion of other transporters which were not classified into the five efflux superfamilies primarily consist of transporters for ion exchange amino acid transport and nutrient uptake/export. These include sugar phosphotransferase systems (PTS) the amino acid-polyamine-organocation (APC) family and the resistance to the homoserine/threonine (RhtB) family.

The efflux pump profile of *S.* sp. HRI was similar to the ATCC strain in that the vast majority of the displayed transporters belong to the ABC superfamily (Figure 1). Similarly, the second most abundant efflux transporter family was the MFS transporters (Figure 1). The DMT superfamily comprised a small proportion of the total transporter complement (Figure 1). Biocide-specific SMR subfamily transporters were present, such as QacE, SugE, and EmrE conferring elevated disinfectant tolerance. The MOP superfamily and the RND superfamily were represented the least in the total repertoire, lacking any biocide-specific transporters (Figure 1). A large proportion of transporters were unable to be classified into the five efflux superfamilies (Figure 1). Some of the more abundant transporters present in this grouping were from the APC family, the RhtB family, and the solute: sodium symporter (SSS) family. Many of the unclassified transporters function as transporters for molecules, such as threonine, serine, ions, and metals.

Resistance transporters in *S. marcescens* subsp. *marcescens* ATCC 13880 and *S.* sp. HRI constituted a small proportion of the total transporters identified (Tables S1 and S2). The ATCC strain had resistance transporters from all five efflux superfamilies (Figure 2). The MFS family represented the greatest proportion of resistance substrate/function efflux pumps. The DMT superfamily had the second-highest abundance of resistance efflux pumps, followed by the RND and the ABC superfamilies which shared an equal abundance of transporters implicated in resistance. The identified members of the RND superfamily were permease/membrane fusion subunits mostly predicted to have multidrug resistance functioning. Many of the ABC superfamily members were uncategorized according to the BLAST results. Furthermore, several transporters were uncharacterized, such as YbhF/YbhS, but these proteins had multidrug functioning predictions (Table S1). The MOP superfamily consisted of a very small proportion of the transporters, mainly from the multidrug and toxin extrusion (MATE) subfamily. Identified transporters from the MATE subfamily included NorM and MdtK. The aromatic acid exporter (ArAE) family consisted of the largest proportion of transporters not grouped into the five efflux superfamilies. These transporters were mainly predicted to have fusaric acid transport functioning (Table S1). The proteobacterial antimicrobial compound efflux (PACE) family is one of the two new transporter families that has recently been identified [16]. Two transporters from this family were predicted; however, no protein identity was elucidated from the amino acid sequences (Table S1).

Interestingly, *S.* sp. HRI had fewer resistance transporters than the ATCC strain (Figure 2). However, the predicted resistance superfamily grouping ratio was similar for both isolates. The MFS family had the greatest abundance of resistance proteins in the HRI isolate, mainly consisting of transporters of the multiple drug transporter (Mdt) family (Table S2). The DMT and RND superfamilies shared equal numbers of transporters and represent the second most abundant grouping of resistance proteins predicted. Mdt and RarD proteins were predicted to have multidrug and chloramphenicol substrate functioning, respectively (Table S2). The ABC superfamily and the MOP superfamily composed a small number of the resistance transporters. The ABC superfamily was predicted to have multidrug function proteins, such as YbhR and YbhF. Additionally, a singular macrolide-specific protein, MacA, (Table S2), and two MATE subfamily transporters, MdtK and EmmdR/YeeO, with multidrug substrate functioning were predicted (Table S2).

3.2. Minimum Inhibitory Concentrations for Isolates

The mean disinfectant concentrations for MIC results are presented in bar charts to better visualize the differences between the *Serratia* isolates (Figures 3–5). Susceptibility tests for BAC showed that the MIC for the HRI isolate was 49-fold higher than the ATCC strain. Thus, according to the criteria required to classify an organism as resistant, the HRI isolate is resistant to BAC. The addition of RSP increased the susceptibility of both *Serratia* isolates to disinfection (Figures 4 and 5). The mean disinfectant concentration that the ATCC strain could tolerate was reduced 5-fold, and the HRI isolate disinfectant tolerance was reduced 42-fold with RSP supplementation (Figures 4 and 5). Unexpectedly, the addition of CCCP caused significant increases in the mean disinfectant concentrations that the isolates could tolerate. BAC disinfection with CCCP supplementation resulted in a 2-fold and 4-fold increase in the mean disinfectant concentration tolerance for the ATCC and HRI isolates, respectively (Figures 4 and 5).

The mean disinfectant concentration of DDAC the HRI isolate was able to tolerate was lower than the mean disinfectant concentration of BAC. However, the ATCC strain displayed higher tolerance toward DDAC than BAC. Like BAC disinfection, the supplementation of RSP increased the *Serratia* strains' susceptibilities toward DDAC disinfection. The susceptibility towards DDAC increased 9-fold and 6-fold for the ATCC and HRI strains, respectively (Figures 4 and 5). CCCP supplementation during DDAC disinfection increased susceptibility 4-fold against the ATCC strain (Figure 5). A paradoxical decrease in susceptibility was reported when DDAC + CCCP was used in combination testing against the HRI

isolate. The mean disinfectant concentration that the HRI isolate tolerated increased 1-fold after CCCP supplementation (Figure 4).

The HRI isolate was able to tolerate a significantly higher concentration of HyperCide® disinfectant than the ATCC strain, as depicted in Figure 3. RSP supplementation increased susceptibility toward HyperCide®, producing 4- and 11-fold decreases in the mean disinfectant concentration of the ATCC and HRI strains were able to tolerate, respectively (Figures 4 and 5). The decline seen when RSP was added indicates that efflux pump activity is responsible, at least in part, for the tolerance toward HyperCide® in both isolates. The addition of CCCP to HyperCide® treatment caused mixed results for both isolates. CCCP supplementation increased the HRI isolate's susceptibility and decreased the ATCC strain's susceptibility to HyperCide® disinfection (Figures 4 and 5).

4. Discussion

Bacterial resistance against QACs and other disinfectants has been reported in many different bacterial species, including ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* sp.) [17,18]. However, resistance to disinfectants is not restricted to these species only, resistance has been observed in *Listeria monocytogenes* and *S. marcescens* in the food and medical industries, respectively [19,20]. Both chromosomal- and plasmid-encoded efflux pumps confer resistance against disinfectants [21,22]. Efflux machinery is well-adapted to reduce/prevent cellular damage in environments with hazardous chemicals and toxic metabolic waste products [23]. Active transport of any hazardous compounds out of the cell decreases the intracellular concentration of the compounds, allowing for increased cell survivability [21].

During this study, the transporters of each isolate were predicted using an annotation pipeline that uses the transporter classification system to provide an identity and substrate/function prediction for each transporter (Figure 1). Both isolates had few transporters with resistance substrate/function predictions (Figure 2). However, the numerical count of efflux pumps cannot be directly correlated to an expected resistance phenotype since the acquisition/activation of a single gene for an efflux pump or multidrug efflux pump can make a cell less susceptible [24].

The ATCC strain was found to have MFS efflux pumps, such as EmrB, MdtG, MdtL, and MdtD, implicated as multidrug transporters with antibiotic resistance capabilities [25]. The DMT superfamily resistance efflux pumps were mainly from the SMR subfamily, such as MdtJ, SsmE/QacE, and SugE (Table S1). The SugE transporter was one of the three possible QAC-specific transporters to be identified [26]. The other potential QAC-specific transporters are QacE from the DMT superfamily and QacA from the MFS. These plasmid-borne efflux pumps are well-studied in *S. aureus* but can be spread to other species through horizontal gene transfer [27,28]. The RND superfamily had efflux pumps, such as AcrD, SdeB, MexW/MexI, and MdtB/MuxB, all with multidrug substrate/function (Table S1). Most of the members of the ABC superfamily were uncharacterized in function according to BLAST, such as YbhF/YbhS (Table S1). The MOP flippase superfamily consisted of a very small proportion of the transporters mainly from the MATE subfamily. This subfamily of transporters is well known to contribute to multidrug resistance with well-studied transporters such as MdtK, DinF, and NorM identified (Table S1) [29,30]. Two transporters from the PACE family were predicted; however, the BLAST results could not provide the transmembrane protein identity. Generally, proteins from the PACE family transport biocides, such as chlorhexidine, suggesting these proteins could be involved in disinfectant tolerance seen in the ATCC 13880 strain [31].

Surprisingly, *S.* sp. HRI had fewer resistance transporters compared to the susceptible ATCC strain (Figure 2). The SMR subfamily proteins predicted include QacE, SugE, EmrE, and SsmeE. These proteins are well established to confer resistance to disinfectants, specifically QacE and SugE which have specificity for QACs (Table S1) [26,32]. The *Serratia* isolates shared all the predicted QAC-specific efflux pumps, suggesting that these efflux pumps

are not responsible for the higher level of resistance observed in *S.* sp. HRI compared to the type strain. A comparison of the efflux pumps revealed that the HRI isolate had two efflux pumps absent from the ATCC strain. These pumps are the SMR transporter EmrE and the MFS transporter SmfY. (Table S1). EmrE has been shown to mediate QAC tolerance in *L. monocytogenes*, and SmfY is a multidrug efflux pump characterized in *S. marcescens* conferring tolerance to a range of antimicrobial agents [20,33]. Although these pumps could be responsible for the resistance of the HRI isolate, differential expression of the shared efflux pumps between the two isolates could also add to the vastly different levels of susceptibility.

SdeB and SdeY are members of the RND superfamily. Both efflux systems were predicted in both isolates. These efflux systems have been previously identified as part of the SdeAB and SdeXY systems in *S. marcescens* [24,34]. Ethidium accumulation assays using CCCP in *E. coli* clones carrying *sdeXY* genes showed that SdeXY is an energy-dependent drug efflux pump resulting from the inhibition that CCCP induced [24].

The MFS pump EmrB was predicted in both isolates. This monomeric efflux protein can form a tripartite system with EmrA and TolC; however, it was predicted to function exclusively in HRI [35]. The transcriptional repressor, EmrR, negatively regulates the expression of the *emrAB* operon by binding the promotor region [36]. There is limited knowledge of the functionality of EmrR to regulate *emrB* in its monomeric form. The substrate specificity of EmrAB-TolC has been shown for antibiotics, a set of unrelated antimicrobial substances that act as uncouplers of the PMF and hydrophobic compounds. CCCP is a substrate of EmrAB-TolC, which directly binds to EmrR to induce the expression of *emrAB* [37]. Therefore, if EmrB has substrate specificity for CCCP and EmrR regulates EmrB, the inactivation of EmrR would increase the functionality of EmrB reducing the uncoupling of the PMF. This activation and subsequent activity of EmrB could explain the decreased susceptibility/paradoxical growth seen when CCCP is supplemented.

The MICs were determined for QACs and HyperCide® in the presence and absence of EPIs. The MIC results displayed statistically significant differences between the MIC of the HRI and ATCC strains (Figure 3). Classifying the HRI isolate as resistant based on testing against the closest related type strain. Standard EPIs were chosen based on functionality, with RSP blocking the transporter channels of certain efflux families, such as RND, MF, and ABC superfamilies [38]. The susceptibility changes show that some specific efflux pump activity is present in conferring disinfectant resistance/tolerance. RSP supplementation significantly reduced the MICs for all disinfectants tested (Figures 4 and 5). From the results, efflux pump(s) from the ABC, MF, or RND superfamilies is/are responsible for QAC and HyperCide® associated resistance.

The protonophore CCCP acts by uncoupling oxidative phosphorylation which disrupts the ionic gradient of bacterial membranes [39]. All the efflux superfamilies, excluding the ABC superfamily, utilize the PMF as part of the cellular metabolism [40]. The effect of CCCP has been shown to lower the MIC of various biocides tested, including BAC, and therefore increase susceptibility [41]. The same effect has been observed with ciprofloxacin as the addition of CCCP decreased MIC values [42]. However, this study showed significantly higher MIC values for *Serratia* species after the supplementation of CCCP to BAC and HyperCide®. Significantly these contradictory decreases in the susceptibility seen when CCCP is supplemented counters the theoretical stance that eliminating energy supply to the efflux pumps via the PMF will inactivate the proteins, thereby increasing the susceptibility to disinfection.

Similar results were observed in a study using a different efflux inhibitor phenylalanine-arginine β-naphthylamide (PAβN), which resulted in paradoxical effects on bacterial inhibition not related to solubility problems [43]. Similarly, in this study, no solubility problems were encountered during the supplementation of CCCP. The Eagle effect could be responsible if the loss of activity cannot be attributed to inconsistent solubility. The Eagle effect is defined as the paradoxical reduced killing of microorganisms at antibiotic concentrations higher than the optimal bactericidal concentration (OBC) unrelated to the solubility [44].

This effect has been described for Gram-positive, Gram-negative, and mycobacteria exposed to numerous antibiotic classes with diverse chemical structures, cellular targets, and sites of action [45–47]. Generally, cell wall-active agents are commonly associated with a paradoxical effect. Antibiotics such as penicillin inhibit protein synthesis to the extent that prevents growth necessary for the drug's lethal effects to occur. Many reports describing the Eagle effect have implicated that the action of autolysins, β-lactamases, and reactive oxygen species (ROS) contribute to the outcome depending on the test antibiotic [48–50]. The Eagle effect has been described for antibiotic treatment and EPI-supplemented antibiotic treatment [51]. The Eagle effect has not yet been implicated in paradoxical reduced killing after EPI-supplemented disinfection.

In conclusion, the results demonstrate that the use of EPIs can be used to improve the in vitro susceptibility of *Serratia* strains to various disinfectants. This work shows that efflux pumps provide resistance to *S.* sp. HRI to test disinfectants within the given contact time and that specific superfamilies predicted can be linked to the resistance. Further investigation is required to determine if efflux pumps are involved in the long-term resistance capabilities of the *Serratia* strains. Additionally, further studies should evaluate the effect of these inhibitors in combination with other antimicrobials and their impact on different microorganisms. An interesting avenue for further study would be to couple the use of EPIs with favorable pharmacokinetics and toxicity profiles in addition to disinfectants in combination therapy techniques where MDR isolates are common. This combination therapy approach could have massive implications in battling MDR profiles in bacteria and the medical industry rife with "superbugs" resistant to many antimicrobials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10112257/s1, Table S1: Resistance transporter classes in *S. marcescens* subsp. *marcescens* ATCC 13880; Table S2: Resistance transporter classes in *S.* sp. HRI.

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2.3 Supplementary material

Table S1: Resistance transporter classes in *Serratia marcescens* subsp. *marcescens* ATCC 13880

TransAAP Identification Number	Subfamily	Substrate/Function
ABC		
62389.1	Inner membrane permease (YbhR)/transporter permease	Multidrug
62390.1	Inner membrane permease (YbhS)/transporter permease	Multidrug
62391.1	ATP-binding protein (YbhF?)/ATP-binding protein	Daunorubicin/multidrug
64836.1	ATP-binding protein/permease	Multidrug
66106.1	Permease	Multidrug
66107.1	Permease	Multidrug/daunorubicin resistance
64109.1	Substrate-binding protein	Aliphatic sulfonate resistance
64989.1	ATP-binding protein (DrrA)	Daunorubicin/doxorubicin resistance
ArAE		
62823.1	Inner membrane protein (yccS?)	Fusaric acid
63253.1	FUSC family protein	Fusaric acid
64184.1	Inner membrane protein (yeeA?)	Fusaric acid
65233.1	Efflux pump subunit (AaeB)	p-hydroxybenzoic acid
65411.1	Inner membrane protein (yccS?)	Fusaric acid
66503.1	FUSC family protein	Fusaric acid
DMT		
62429.1	Permease/inner membrane protein	Multidrug
62530.1	Inner membrane protein (yedA?)	Multidrug/uncharacterised
63606.1	SMR transporter (SsmE/QacE)	Multidrug
63969.1	SMR transporter (MdtI)	Multidrug/spermidine
63770.1	SMR transporter (MdtJ)	Multidrug/spermidine
66021.1	SMR transporter (SugE)	Quaternary ammonium compounds
63204.1	4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase subunit (ArnE)	Polymyxin

63205.1	4-amino-4-deoxy-L-arabinose-phosphoundercaprenol flippase subunit (ArnF)	Polymyxin
64117.1	Permease (RarD)	Chloramphenicol
65878.1	Permease (RarD)	Chloramphenicol
MFS		
62207.1	fsr	Fosmidomycin
62550.1	stp_1	Spectinomycin/tetracycline
62255.1	Bcr/CflA transporter	Multidrug
62533.1	Rv1634/MT1670 transporter	Multidrug
62909.1	MdtG transporter	Multidrug
63237.1	Bcr/CflA transporter	Multidrug
63376.1	MdtG transporter	Multidrug
63491.1	bmr3_2 transporter	Multidrug
63594.1	Multidrug resistance protein D	Multidrug
63699.1	Multidrug transporter SmfY	Multidrug
63945.1	MdtL transporter	Multidrug
63798.1	MdtH transporter	Multidrug
64227.1	Bcr/CflA transporter	Multidrug
64545.1	MdtD transporter	Multidrug
64742.1	EmrB permease subunit	Multidrug
65598.1	EmrD transporter	Multidrug
65677.1	MdtD transporter	Multidrug
65770.1	MdtA transporter	Multidrug
63314.1	EmrB/QacA transporter	Unknown/quaternary ammonium compounds
63510.1	Tet(A)/Tet(B)/Tet(C) transporter	Tetracycline
MOP		
62464.1	MATE family efflux transporter (NorM?)	Unknown/uncharacterised
63233.1	MdtK transporter/MATE family efflux transporter	Multidrug
64073.1	EmmdR/YeeO MATE family efflux transporter	Multidrug
65299.1	DNA-damage-inducible protein/MATE family efflux transporter (DinF)	Oxidative stress/bile salts
PACE		
62413.1	Efflux transporter	Unknown
64492.1	Efflux transporter	Multidrug/biocide

RND		
62187.1	Permease subunit (SdeY) HAE1	Multidrug
62844.1	Permease subunit (MexW/MexI) HAE1	Multidrug
62998.1	Permease subunit (SdeB) HAE1	Multidrug
63489.1	Permease subunit HAE1	Multidrug
64491.1	Permease subunit (AcrD) HAE1	Multidrug/aminoglycoside
64543.1	Permease subunit (MdtB/MuxB) HAE1	Multidrug
64544.1	Permease subunit (MdtC) HAE1	Multidrug
64571.1	Membrane fusion protein (MdtN)	Multidrug

Table S2: Resistance transporter classes in *Serratia* sp. HRI

TransAAP Identification Number	Subfamily	Substrate/Function
ABC		
2607	Permease/ATP-binding protein	Multidrug
2638	Membrane protein	Daunorubicin
5693	Macrolide-specific efflux protein (MacA)	Macrolides
4956	ATP-binding protein	Multidrug
ArAE		
4216	FUSC family protein	Fusaric acid
DMT		
1271	Quaternary ammonium compound efflux SMR transporter (SugE)	Quaternary ammonium compound
5131	SMR Transporter (QacE)	Quaternary ammonium compound
1417	Permease (RarD)	Chloramphenicol
3981	Permease (RarD)	Chloramphenicol
2051	DMT transporter	Multidrug
3542	Transporter Subunit (MdtI)	Multidrug/spermidine
3543	Transporter Subunit (MdtJ)	Multidrug/spermidine
4058	SMR Transporter (EmrE)	Multidrug
5428	SMR Transporter (SsmE)	Multidrug
4864	Uncharacterised/Inner membrane protein (YedA)	Drug/metabolite
MFS		

1509	Translocase (MdtA)	Multidrug
1609	Transporter subunit (MdtD)	Multidrug
1711	Transporter (EmrD)	Multidrug
2294	Transporter subunit (MdtD)	Multidrug
2493	Transporter Permease (EmrB)	Multidrug
3517	Transporter (Bcr/CflA)	Multidrug
3581	Transporter (MdtH)	Multidrug
3830	Transporter	Multidrug
4140	Transporter (EmrB/QacA)	Multidrug
4235	Transporter (Bcr/CflA)	Multidrug
4616	Transporter (MdtG)	Multidrug
5103	Transporter (Bcr/CflA)	Multidrug
5179	Transporter (MdtG)	Multidrug
5418	Protein D	Multidrug
71	Multidrug efflux transporter (SmfY)	Multidrug
4867	Probable transporter (Rv1634/MT1670)	Multidrug
1920	Transporter (Bcr/CflA)	Bicyclomycin
500	Efflux system (fsr)	Fosmidomycin
5309	TCR/Tet Family transporter	Tetracycline
5310	Tet(A)/Tet(B)/Tet(C) family transporter	Tetracycline
MOP		
3909	EmmdR/YeeO family MATE transporter	Multidrug
4248	MATE family efflux transporter (MdtK)	Multidrug
PACE		
2148	Multidrug/biocide efflux PACE transporter	Multidrug/biocide
RND		
2230	Aminoglycoside/multidrug efflux RND transporter permease (AcrD)	Multidrug/aminoglycoside
2291	Multidrug efflux RND transporter periplasmic adaptor subunit (MdtA/MuxA)	Multidrug
2292	Multidrug efflux RND transporter permease subunit (MdtB/MuxB)	Multidrug
2293	Multidrug efflux RND transporter permease subunit (MdtC)	Multidrug
4544	Multidrug efflux RND transporter permease subunit (SdeB)	Multidrug
4688	Multidrug efflux RND transporter permease subunit (MexW/MexI)	Multidrug

522	Multidrug efflux RND transporter permease subunit (SdeY)	Multidrug
3449	Cu(+)/Ag(+) efflux RND transporter permease subunit (SilA)	Heavy metals
3450	Cu(+)/Ag(+) efflux RND transporter permease subunit (SilB)	Heavy metals
4637	Putative membrane fusion protein (EtsA)	Macrolides

2.4 References

It is important to note that the references included in the article in this thesis will not be repeated in the reference section for this chapter.

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Chapter 3: Long-term susceptibility and degradation activity investigation of *Serratia* species

Abstract

Disinfection protocols and biosecurity are vital parts of effective microbial disease control. Resistance development over long-term exposure to antimicrobial compounds is threatening a variety of settings. Efflux pumps are renowned as significant resistance mechanisms allowing bacteria to resist disinfection. This study aimed to ascertain how a multidrug-resistant *Serratia* sp. HRI isolate could withstand long-term disinfection using a well-known quaternary ammonium compound (QAC), didecyldimethylammonium chloride (DDAC). Furthermore, the degradation activity of the strain was investigated using liquid chromatography tandem mass spectrometry (LC-MS/MS). Time-kill assays were conducted in the presence and absence of efflux pump inhibitor (EPI) reserpine (RSP) to elucidate the role of efflux pumps during long-term disinfection. The supplementation of RSP significantly reduced the tolerance capabilities of the HRI strain for 1 532 mg/L and 383 mg/L DDAC concentrations. The LC-MS/MS investigation highlighted the potential DDAC degradation pathways that *Serratia* sp. HRI possesses. This study provides evidence that *Serratia* sp. HRI could possess multiple mechanisms of disinfectant resistance. Furthermore, these different mechanisms are possibly recruited based on the disinfectant concentration.

3.1 Introduction

Effective disinfection strategies rely on the proper understanding of the mechanism of action and at which concentrations the chosen disinfectant exerts its bactericidal or inhibitory effects

(Lambert and Pearson 2000). Understating the factors that contribute towards ineffective disinfection is critical to curbing the proliferation of bacterial populations. Furthermore, ineffective disinfection could promote the emergence of resistance in bacteria. Non-volatile disinfectants may pollute unintended environments at sub-inhibitory concentrations (Ribič et al. 2020a). This promotes further selective pressure for resistance in clinically/industrially unrelated bacteria to these low levels of contaminating compounds which pollute various environments (Ribič et al. 2020a; Nordholt et al. 2021). This case has been made more relevant due to the persistent use of disinfection/sanitiser chemicals during the SARS-CoV-2 pandemic (Hora et al. 2020; Pedreira et al. 2021). The effects of practical interfering substances, such as organic matter, and bacterial biofilm formation can even lead to supra-minimum inhibitory concentration (MIC) treatment being ineffective (Araújo et al. 2013). The non-volatile, cost-effective, and single-use cleaning capabilities of quaternary ammonium compounds (QACs) in low-soiling environments urge the need to understand the sub-MIC kinetics (Kampf 2018).

QACs are often incorporated into the industry, household, and cosmetic products. QACs incorporated in the aforementioned settings serve no antibacterial purpose but are rather added as emulsifiers, softeners or floating agents (Zhang et al. 2015). The promoting factor of QACs is that these agents are considered safe for users and consumers, causing minimal corrosion to applied surfaces in comparison to oxidative disinfectants. The selective pressure exerted but sub-inhibitory QAC concentrations relate to increased bacterial resistance. When exposed to the QACs, the bacteria must achieve some way to ergonomically overcome/survive the environmental stress. This can be achieved by acquiring new resistance mechanisms from the surrounding population or mutation of intrinsic mechanisms. Sub-MIC concentrations can be achieved by the following:

i) incorrect dilution calculation for the working concentrations of a product from a concentrated form of the product.

ii) use of expired or incorrectly stored disinfectants.

iii) inadequate removal of organic matter before disinfection treatment (which interferes with disinfectant activity because of the organic material).

iv) irregular spreading of disinfectant during treatment (Capita et al. 2019).

Within the presence of low-level QACs, microbial adaptation to QACs can lead to tolerance to other antimicrobial substances, including other disinfectants or antibiotics (Soumet et al. 2016; Capita et al. 2019). Generally, several mechanisms of bacterial adaptation or tolerance are involved in conferring reduced susceptibility, including cell growth modulation, efflux pump activity and cell membrane adaptations (Jennings et al. 2015; Gadea et al. 2017; Ribič et al. 2020b). However, tolerance development to QACs requires more understanding (Morrison et al. 2019).

Active efflux is one of the primary mechanisms of the disinfectant resistance (Hassan et al. 2010). With this mechanism, bacteria can extrude the disinfectant out of the cell using membrane-embedded transport proteins. This reduces the intracellular concentration of disinfectant to a non-threatening level (Piddock 2006). Five main families of efflux pumps identified as responsible for transport implicated in the antimicrobial resistance (Piddock 2006; Hassan et al. 2010). It is unclear whether these transporters are recruited as a primary means to combat disinfectants or whether they are temporary mechanisms buying time for other resistance mechanisms to be introduced. A better understanding of how QACs interact with bacteria at sub-MIC conditions and which resistance mechanisms are used to withstand low concentrations requires further investigation.

In vitro, time-kill assays can be used to elucidate the extent of antibacterial activity and are recognised as being valuable tools for the characterisation of new agents, but can also be beneficial for uncovering how combinations of substances interact (NCCLS 1999). Generally,

these assays can be used to distinguish bacteriostatic from bactericidal antibiotic concentrations, but this can also be applied to disinfectants (NCCLS 1999). Given the nature of the time-kill assay and bacterial resistance development, over time, an initial decrease in colony counts may result in increases due to the selection of resistant mutants, inactivation of antimicrobials or regrowth of susceptible cells (Layte et al. 1984). Although the inactivation of antibiotics by chemical interaction is well-known, the chemical inactivation of disinfectants is less well studied (Blair et al. 2015). Determining if disinfectants can be inactivated by chemical alterations caused by bacterial metabolic reactions or other reaction pathways could provide insight into coupled resistance mechanisms linking efflux and chemical inactivation.

Previously, the interaction of *Staphylococcus aureus* with a QAC resulted in alterations in the morphology of the bacteria (Jansen et al. 2013). Trace amounts of the QAC were detected within the bacterial cells. The QAC concentration was very low within the cells, hence the exact levels could not be definitively concluded. It was hypothesized that the bacteria altered the structure of the disinfectant prior to efflux out of the cell (Jansen et al. 2013). Liquid-chromatography-mass spectrometry (LC-MS) is a common technique that can be used to analyse QAC presence in environmental samples (Bassarab et al. 2011). LC-MS is chosen above alternative types of liquid chromatography (LC) for environmental analysis because it has greater detection sensitivity (Castro et al. 2000). LC is a technique that separates a sample into individual components or fragments allowing the mass spectrometer (MS) to detect and quantify the analyte of interest (Agilent Technologies 2001). Once separated, on the chromatography column, the MS ionises the intact molecules into a charged state, which breaks the molecule into various fragments. The fragmented ion is identified based on their mass-to-charge ratio *(m/z)* (Agilent Technologies 2001; Pitt 2009). Therefore, using the *m/z* ratio QACs can be detected, and the sample identification can be elucidated allowing any structural changes to be identified.

This study aimed to determine the extent of efflux pump-mediated long-term disinfectant resistance. Additionally, the potential metabolic activity concerning QAC degradation was also investigated. The objectives were to compare two types of bacterial counting methods used in downstream time-kill assays. Furthermore, comparing the *in vitro* activity of sub-MIC concentrations of QAC disinfectant didecyldimethylammonium chloride (DDAC) alone and in combination with known efflux pump inhibitor (EPI) reserpine (RSP) in time-kill assays against a resistant *Serratia* isolate. Furthermore, liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to quantify the uptake and possible alteration in the chemical structure of a QAC disinfectant after exposure to resistant *Serratia* isolate.

3.2 Materials and methods

3.2.1 Chemical agents

Didecyldimethylammonium chloride (DDAC) in liquid form (80% v/v, ICA International Chemicals, Stellenbosch, South Africa) was obtained. The concentrations used were determined based on the recorded minimum inhibitory concentration (MIC) results presented in Chapter 2 [published manuscript] of this thesis. Before each experimental use, fresh dilutions of each disinfectant were made to ensure the proper functionality of the agents. RSP (Sigma-Aldrich, St. Louis, MI, USA) was prepared at a stock concentration of 100 µg/ml. Distilled water was available at the Department of Microbiology and Biochemistry and double-distilled water was obtained from the Department of Plant Sciences at the University of the Free State.

3.2.2 Bacterial strain

A highly resistant *Serratia* species was isolated from a DDAC-based disinfectant bottle (1% v/v) at room temperature at the University of the Free State in South Africa (Mc Carlie et al.

2020). The strain was aerobically grown at 37 ºC for 18-20 hours in Tryptic Soy Broth (TSB) or on Tryptic Soy Agar (TSA) for routine bacterial cultivation.

3.2.3 Drop plate vs. spread plate method

A culture of *Serratia* sp. HRI was prepared by aerobic growth at 37 ºC for 18-20 hours in TSA. A 0.5 McFarland inoculum was prepared in sterile PBS before dilution for the 6 x 6 drop plate procedure. Briefly, each well of the 96-well plate was filled with 180 µl of sterile distilled water. Thereafter, 20 µl of the standardised sample was added to the first column of the plate and serially diluted 10-fold using a multichannel pipette by transferring 20 µl into each successive column. Thereafter, six replicates of 10 µl (ideal for the spacing between multichannel pipette tips) from six selected dilutions (10^{-2}–10^{-7}) were plated onto TSA using a multichannel pipette (Chen et al. 2003a). The plates were allowed to dry, and then placed into an incubator at 37 ºC for 24 hours. The colonies were enumerated after the incubation where a range of 3-30 colonies was established. Both comparison methods consisted of at least three technical replicates and four biological replicates.

3.2.4 Time-kill assays

Time-kill analyses were performed by culturing *Serratia* sp. HRI in BHI medium with the addition of a dilution range of antimicrobial concentrations from 12 259 mg/l to 12 mg/l of DDAC. These DDAC concentrations were chosen to investigate supra-inhibitory, inhibitory, and sub-inhibitory levels against the HRI isolate. Additionally, an efflux pump inhibitor (EPI) was added to determine the combinatory effects of DDAC on bacterial susceptibility. A 0.5 McFarland inoculum of *Serratia* was prepared in sterile (phosphate-buffered saline) PBS by picking one to two colonies from TSA-grown cultures incubated for 18-20 hours at 37 ºC. Firstly, the PBS inoculum was diluted, 30 µl in 15 ml pre-warmed antibiotic-free brain heart infusion (BHI) medium and 90 µl was dispensed per well of a round bottom 96-well plate. The

96-well plates were incubated for 4 hours shaking at 150 rpm, 35 ºC. After the pre-incubation process, at time point t = 0 h, 10 µl of DDAC (or PBS) was added to each well containing 90 µl sample. This resulted in eight identical rows (one row per time point) containing bacteria exposed to 11 different DDAC concentrations and one untreated control (one column per DDAC concentration for 11 columns and one control column). Colony forming units per ml (CFU/ml) readings were taken at eight time points, t = -4, 0, 1, 2, 3, 4, 5 and 6 h, using the drop plate method for efficiency and high throughput potential. All time-kill assays included at least three biological replicates.

Five dilutions from the total eleven tested, were selected for further investigation targeting specific concentrations to fit with supra-inhibitory, inhibitory, and sub-inhibitory classifications. Time-kill assays using these five selected DDAC concentrations, 1532 mg/l, 383 mg/l, 96 mg/l, 48 mg/l and 12 mg/l, were repeated in 250 ml Erlenmeyer flasks. Briefly, 600 µl of 0.5 McFarland bacterial inoculum was diluted into 300 ml pre-warmed antimicrobial-free BHI broth and 22.5 ml per experimental sample was dispensed in 250 ml Erlenmeyer flasks. The flasks were pre-incubated for 4 hours shaking at 150 rpm, 35 ºC. After the pre-incubation period, at time point t = 0 h, 2.5 ml of each of the DDAC concentrations was added to five separate flasks. This produced five flasks with bacteria being exposed to five different DDAC concentrations, one untreated flask containing pre-incubated bacteria (positive control) and one flask containing only BHI (negative control). Colony forming units per ml (CFU/ml) readings were taken at eight time points, t = -4, 0, 1, 2, 3, 4, 5 and 6 h, using the drop plate method on TSA plates. The plates were incubated for 24 hours and then the colonies were enumerated. All the time-kill assays included at least three biological replicates.

Following time-kill assays with DDAC only, RSP was added to determine the extent of efflux pump activity during long-term disinfection. The five concentrations of DDAC used during this investigation were 1 532 mg/l, 383 mg/l, 96 mg/l, 48 mg/l and 12 mg/l. Briefly, 600 µl of 0.5

McFarland bacterial inoculum was diluted into 300 ml pre-warmed antimicrobial-free BHI broth. Thereafter, 22.422 ml of pre-warmed BHI + bacterial inoculum was added into five 250 ml Erlenmeyer flasks representing the five DDAC concentration samples. Additionally, 24.922 ml of pre-warmed inoculum was added to one 250 ml Erlenmeyer flask and 22.5 ml was added to another 250 ml Erlenmeyer flask, representing the EPI control and positive control, respectively. The flasks were pre-incubated for 4 hours shaking at 150 rpm, 35 ºC. After the pre-incubation period, at time point t = 0 h, 2.5 ml of each of the DDAC concentrations was added to five separate flasks containing 22.422 ml BHI and 78 µl of RSP was added to the flask containing 24.922 ml BHI. This produced five flasks with bacteria exposed to five different DDAC concentrations, one RSP treated flask (EPI control), one untreated flask containing pre-incubated bacteria (positive control) and one flask containing only BHI (negative control). At time point t = 3 h, (indicated by the arrow in **Fig 3.3**) RSP was added to a final concentration of 0.3125 µg/ml to each separate flask containing the five DDAC concentrations. CFU/ml readings were taken at eight time points t = -4, 0, 1, 2, 3, 4, 5 and 6 h, using the drop plate method on TSA plates. The plates were incubated for 24 hours and then the colonies were enumerated. All the time-kill assays included at least three biological replicates.

3.2.5 Liquid chromatography with tandem mass spectrometry analysis

Distilled and double distilled water samples were prepared for LC-MS/MS analysis to determine DDAC limits of detection and set up baseline comparison levels. Furthermore, a DDAC dilution series was prepared to further determine the detection and retention time limits of DDAC. Once the limits of detection and the retention times of DDAC were established *Serratia* sp. HRI was standardised to 0.5 McFarland and incubated with DDAC for time points 2, 5, 10, 20, 30, 40, 50 and 60 min. Each of the time point samples were analysed using LC-MS/MS to detect the presence of DDAC by separating the sample components through a stationary phase containing a chromatographic packing material and the mass analyser measured the abundance of each ion in the sample. Standard water samples were prepared

to determine the lower limits of DDAC detection. Additionally, serial dilution standards of DDAC were prepared to determine the retention time and establish the upper detection limits of DDAC. All samples were analysed using an ABSCIEX 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer with a Shimadzu high-performance liquid chromatography stack as a front end. All data acquisition and processing were performed using Analyst 1.5 (AB SCIEX) software.

A total of 20 µl of each extracted sample were separated on a C8 (150 mm x 4.6 mm, Phenomenex) column, in positive ionisation mode, at a flow rate of 300 µl/min using a 15 min gradient from 5% solvent A (H_2O/0.1% formic acid) to 95% solvent B (MeOH/0.1% formic acid). Eluted analytes were ionised by electrospray in the TurboV ion source. The following settings were adjusted on the TurboV ion source; 5500 V ion spray voltage, 400 ºC heater temperature to evaporate the excess solvent, 30 psi nebuliser gas, 30 psi heater gas and 30 psi curtain gas.

The targeted analyses of DDAC were performed using 2 multiple reaction monitoring (MRM) transitions. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the second transition is used as a qualifier. The analyte peak area of the quantifier calculated was used for the relative quantification of the presence of DDAC. The qualifier serves as an additional level of confirmation for the presence of the analyte. The retention time for the quantifier and qualifier transitions needs to be the same. This is necessary to ensure that it is the same molecule generating the qualifier and quantifier measurements. The peak areas of the unknown samples were related to the quantified value using the calibration curve for each analyte, normalised to the peak area of the internal standard.

The MRM transitions were as follows:

Positive ionization mode		
Q1 (m/z)	Q3 (m/z)	Analyte
326.3	186.2	DDAC Fragment 1
326.3	57.1	DDAC Fragment 2

3.2.6 Statistical analysis

The data presented in **Table 3.1** depicts the mean bacterial count (CFU/ml) of the drop plate method compared to the spread plate method (n = 4 biological replicates). This data was analysed using the two-sample t-test assuming equal/unequal variance based upon the f-test for the variance of the two groups. $p < 0.05$ was considered statistically significant. The data in **Fig. 3.2** and **Fig. 3.3** depicts the mean bacterial count (CFU/ml) ± standard deviation (n = 3 biological replicates). In **Fig. 3.2**, each respective time point (t = 0, 1, 2, 3, 4, 5, 6) of the positive control was compared to the corresponding time point for each disinfectant concentration (1 532 mg/l, 383 mg/l, 96 mg/l, 48 mg/l, 12 mg/l). The data was analysed using the two-sample t-test assuming equal/unequal variance based upon the f-test for variance for each respective grouping. $p < 0.05$ was considered statistically significant. In **Fig. 3.3**, each respective time point of the positive control and the EPI control were compared. Thereafter, each disinfectant concentration was compared with (data in **Fig. 3.3**) and without (data in **Fig. 3.2**) RSP addition after t = 3 h. The data in both instances was analysed using the two-sample t-test assuming equal/unequal variance based upon the f-test for variance. $p < 0.05$ was considered statistically significant. All the data was analysed on SAS 9.4.

3.3 Results

3.3.1 Drop plate vs. spread plate method

A comparison between the two agar-based bacterial enumeration methods was conducted to ensure that there is no statistical difference between the two methods. This is important so that the drop plate method can be used for downstream time-kill assays. The results displayed in **Table 3.1** display the mean bacterial counts (CFU/ml) of the two counting methods (n = 4 biological replicates). The two counting methods, according to **Table 3.1**, were not statistically different (p = 0.9); thus, the drop plate method could be utilised as a reliable counting method for the downstream time-kill assays.

Table 3.1: Mean bacterial count (CFU/ml) of the drop plate method compared to the spread plate method.

Replicates	Mean drop plate counts (CFU/ml)	Mean spread plate counts (CFU/ml)
A	4.7×10^9	7×10^8
B	8.5×10^8	4.6×10^9
C	1.3×10^8	1.3×10^8
D	9×10^7	9.6×10^7

p = 0.9

3.3.2 Time-kill assays

A time-kill analysis was conducted to visualise the effects of a range of DDAC concentrations against *Serratia* sp. HRI. Firstly, DDAC concentrations were tested in 96-well plates to elucidate the survivability capacity of *Serratia* sp. HRI. **Fig. 3.1** displays 11 serially diluted DDAC concentrations tested against the *Serratia* strain for 6 hours. Some of these concentrations fall below the recorded MIC value for this strain as determined in the published work presented in Chapter 2. In **Fig. 3.1** the highest concentrations of DDAC (12 259 mg/l and 6 129 mg/l) decrease the mean bacterial counts to below the limit of detection within the first 3 hours of the analysis.

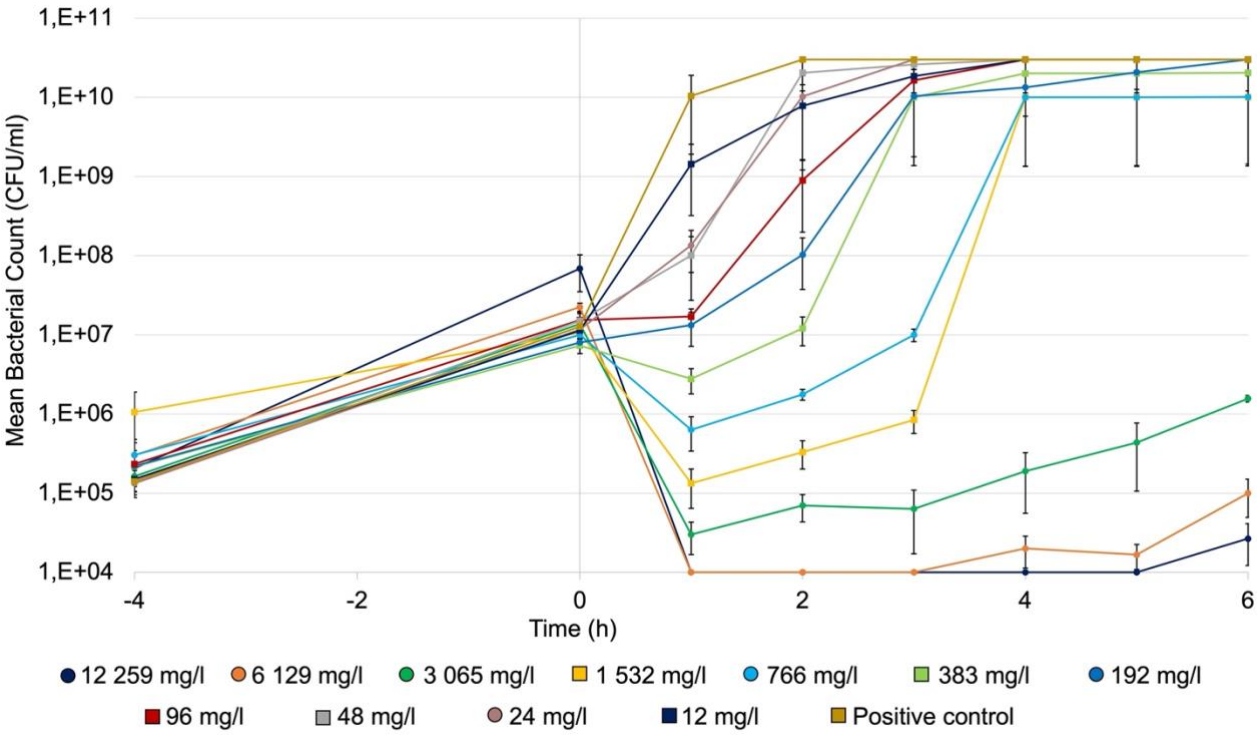


Figure 3.1: Time-kill analysis of mean bacterial counts (CFU/ml) of *Serratia* sp. HRI against various concentrations of DDAC.

The remainder of the DDAC concentrations tested inhibited bacterial growth but not to such an extreme degree. The time-kill assay displayed the expected concentration-dependent decrease in the mean bacterial count recorded. Meaning as the DDAC concentration decreased the bacteria was more capable to withstand disinfection and proliferate. After t = 3 h, the mean bacterial count increased to within the limit of detection for the highest DDAC concentrations (12 259 mg/l and 6 129 mg/l) and gradual increases in bacterial population occurred throughout the analysis (**Fig. 3.1**). DDAC concentrations ranging from 3 065 mg/l to 383 mg/l displayed inhibitory effects on the bacterial population during the first hour of the time-kill analysis. However, from time point t = 1 h onwards the survivability of the bacterial population gradually increased throughout the analysis (**Fig. 3.1**). DDAC concentrations tested closest to the recorded minimum inhibitory concentration (MIC) in the published work in Chapter 2 (192 mg/l and 96 mg/l) both exhibited inhibitory effects on the bacterial population during the first 4 hours of the analysis (**Fig. 3.1**). After t = 4 h, the bacterial population increased to the limit of detection for the remaining time points. DDAC concentrations below the recorded MIC in the published work in Chapter 2 (48 mg/l to 12 mg/l) exhibited minimal inhibitory effects on the survivability of the bacterial population (**Fig. 3.1**). From the addition of the sub-inhibitory DDAC concentrations at t = 0 h, the mean bacterial count increased sub-optimally in comparison to the positive control. However, these bacterial populations eventually reached the maximum limit of detection after 4 hours. In comparison to the bacterial populations exposed to DDAC, the positive control reached the limit of detection at t = 2 h. The square graph points represent the selected DDAC concentrations for downstream efflux pump activity investigation, these include 1 532 mg/l, 383 mg/l, 96 mg/l, 48 mg/l and 12 mg/l (**Fig. 3.1**).

The five DDAC concentrations selected for further investigation and up-scaling in 250 ml Erlenmeyer flasks displayed similar results as those from the 96-well plate assays. Generally, the mean bacterial counts of the up-scaled investigation were higher, due to more beneficial

growth conditions that included increased aeration promoting more prolific growth. **Fig. 3.2** displays the time-kill analysis using the five selected DDAC concentrations in the Erlenmeyer flasks. The addition of DDAC at t = 0 h for all concentrations exhibited inhibitory effects on the proliferation capacity of the bacterial population compared to the positive control (**Fig. 3.2**). This is evident from the differences in mean bacterial counts recorded at each time point. However, very few statistically significant differences were recorded between the mean bacterial counts of the positive control and the DDAC concentrations. The inhibitory effect of 1 532 mg/l was the greatest when considering the decreases induced in mean bacterial count (**Fig. 3.2**). However, the bacterial count for 1 532 mg/l only differed significantly from the positive control bacterial count at t = 4 h. The remainder of the points were not significantly different from the positive control bacterial counts.

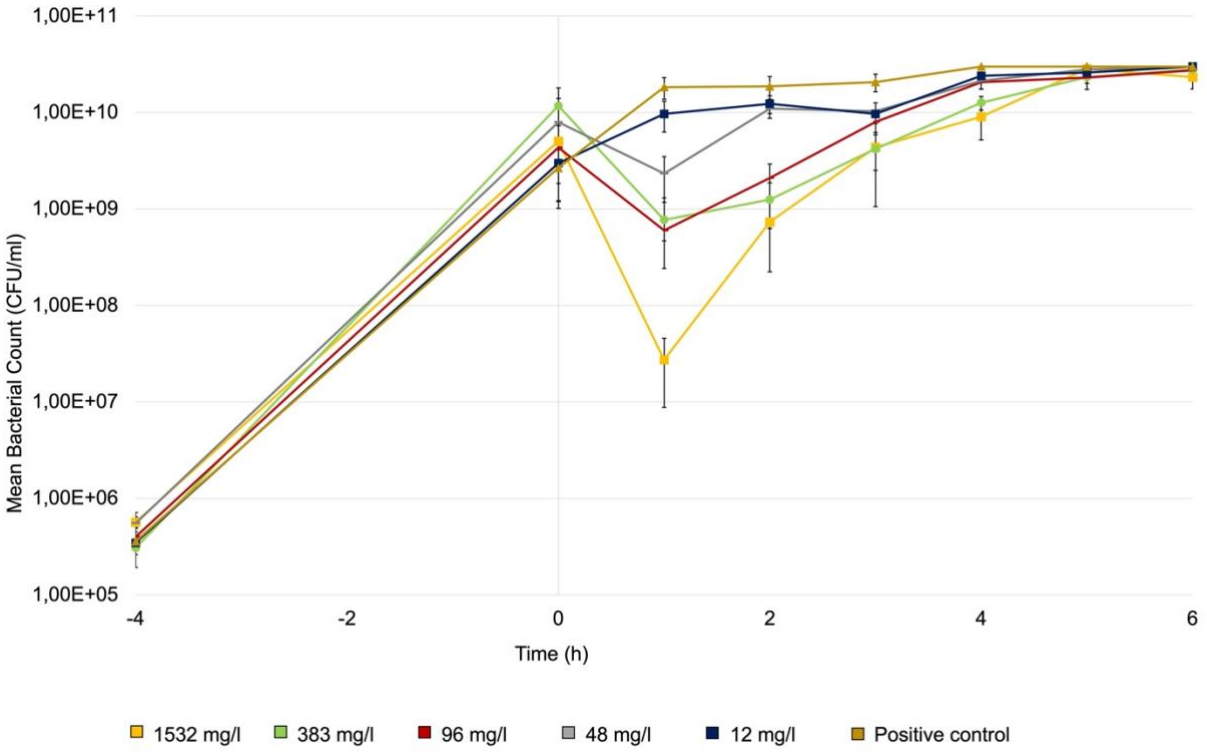


Figure 3.2: Time-kill analysis of mean bacterial counts (CFU/ml) of *Serratia* sp. HRI using five selected concentrations of DDAC.

Graphically, the remainder of the tested DDAC concentrations exhibited less severe inhibitory effects on the survivability of the bacterial populations (**Fig. 3.2**). The mean bacterial counts for the DDAC concentrations 383 mg/l and 96 mg/l had a sharp increase from t = 1 h until t = 4 h, levelling off toward the end of the analysis (**Fig. 3.2**). However, the bacterial counts of 383 mg/l differed significantly from the positive control bacterial counts at t = 3 h and 4 h. Furthermore, the bacterial counts of 96 mg/l differed significantly from the positive control bacterial counts at t = 2 h. The mean bacterial counts for the DDAC concentrations 48 mg/l and 12 mg/l exhibited gradual increases between t = 1 h and t = 4 h (**Fig. 3.2**). These low DDAC concentrations inhibited the growth of the bacteria until t = 5 h, whereafter the bacterial counts corresponded with the positive control (**Fig. 3.2**). Both the mean bacterial counts of 48 mg/l and 12 mg/l DDAC only differed significantly from the positive control counts at t = 1h.

The time-kill kinetics of the DDAC concentrations with RSP addition are displayed in **Fig. 3.3**. The mean bacterial counts of the EPI and positive controls did not differ significantly at any time points throughout the analysis. The lack of significant differences between the two controls reiterates that at the used concentration RSP exhibited no intrinsic inhibitory effect on the bacterial population. The effect that RSP exhibited on the mean bacterial count of the highest DDAC concentration (1 532 mg/l) tested, graphically displayed substantial decreases compared to the controls (**Fig. 3.3**). However, when comparing mean bacterial counts for 1 532 mg/l with and without RSP addition, the addition of RSP only induced significant differences at t = 5 h.

An evident decrease in the mean bacterial count was observed after RSP addition at t = 3 h for the bacterial populations tested against 383 mg/l DDAC (**Fig. 3.3**). This decrease was statistically significant when comparing the mean bacterial counts of the 383 mg/l tested populations with and without RSP addition for time points t = 4, 5 and 6. The remainder of the

DDAC concentrations (96 mg/l to 12 mg/l) exhibited minimal graphical changes in the mean bacterial count after RSP addition (**Fig. 3.3**). Specifically, the differences in the 96 mg/l and 12 mg/l DDAC concentrations' mean bacterial counts with and without RSP addition were statistically insignificant for all time points. Like bacterial counts of the 1 532 mg/l, the mean bacterial counts of the 48 mg/l DDAC concentration with RSP addition only differed significantly from the counts without RSP addition at t = 5 h.

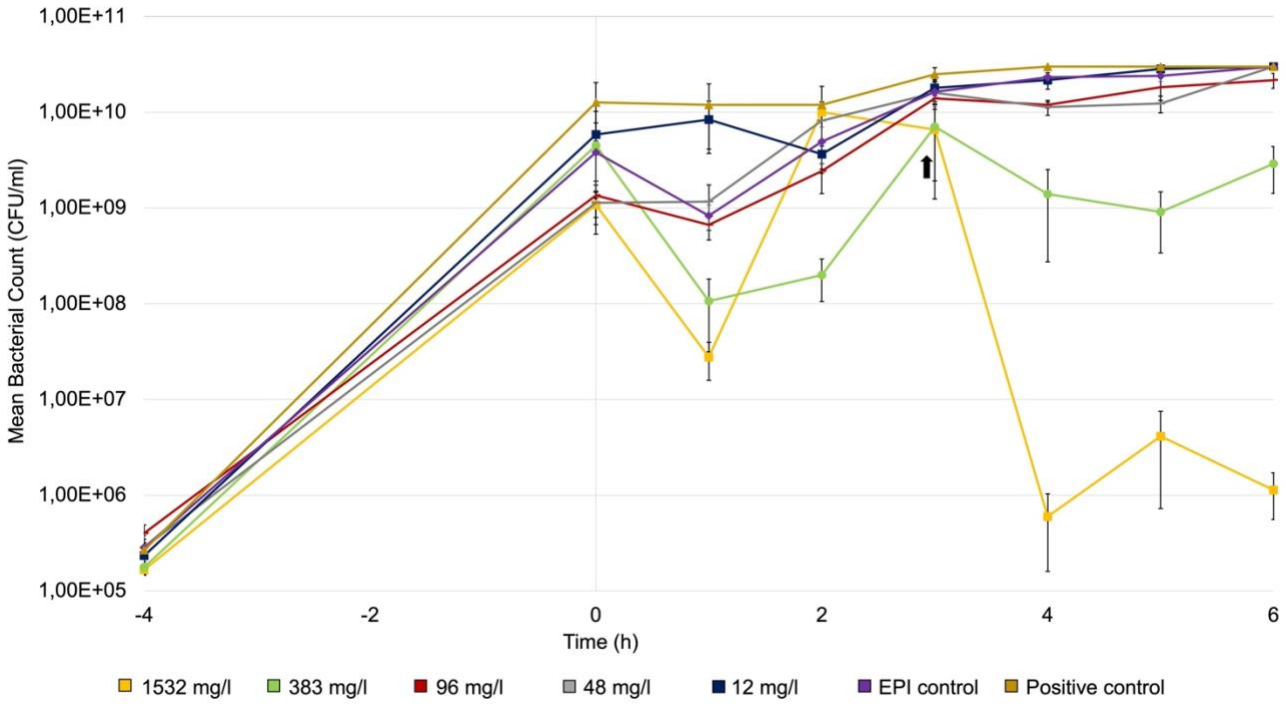


Figure 3.3: Time-kill analysis of mean bacterial count (CFU/ml) of *Serratia* sp. HRI using five selected concentrations of DDAC and addition of RSP (final concentration = 0,3125 µg/ml) at arrow (t = 3 h).

3.3.3 Liquid chromatography with tandem mass spectrometry analysis

The retention time and the lower limit of detection of DDAC were determined from various water samples and a prepared dilution series of DDAC. **Fig. 3.4** shows the chromatogram of one of the DDAC standards prepared in the dilution series. The retention time for DDAC was determined to be approximately 17 min as depicted in **Fig. 3.4**. The DDAC 1 fragment was the largest produced during LC-MS/MS analysis having the greatest analyte peak area at each time point permitting more reliable quantification. Therefore, this fragment was used for relative quantification of DDAC over the experimental time points.

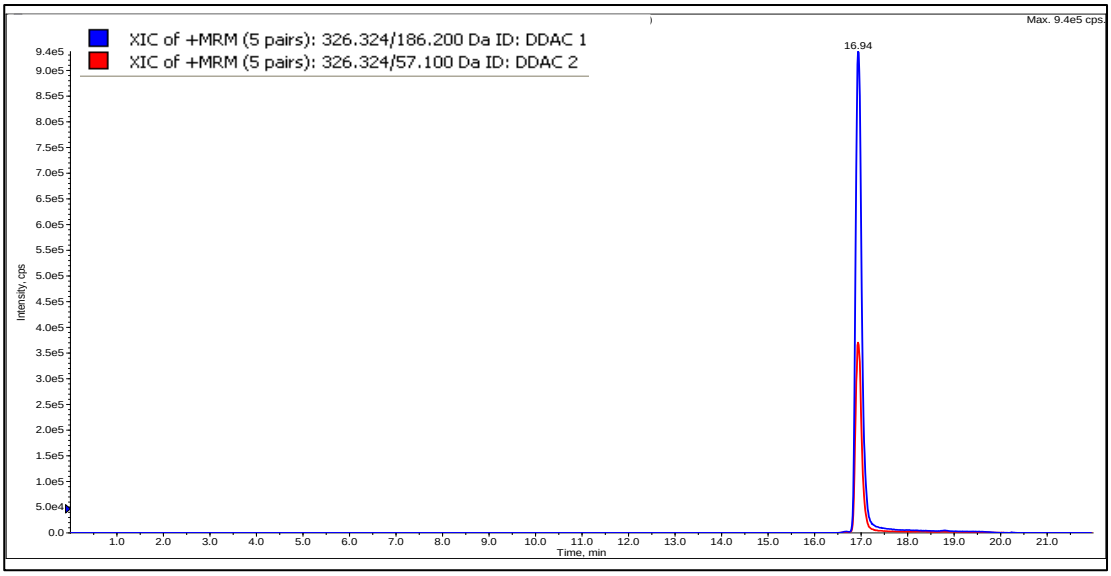


Figure 3.4: Chromatogram of a DDAC standard as obtained from the MS. The retention time for DDAC is approximately 17 min. Different colours represent different fragments of DDAC.

The detection limits of the DDAC were determined by relative quantification using the analyte peak area of each DDAC chromatogram as displayed in **Fig. 3.4**. The raw chromatographic analyte peak areas of the DDAC dilution series were compared to the standardised bacterial samples incubated with DDAC concentrations for various periods of time in a relative quantification approach. Additionally, a standard media control containing only BHI broth was

analysed for comparative purposes and lower limit detect of DDAC. The effect of RSP was not tested in these experiments. **Fig. 3.5** represent the highest concentrations of DDAC quantified being 12 259 mg/l, 6 129 mg/l and 3 065 mg/l, respectively. These DDAC concentrations fall outside the upper limit of the mass analyser's limit of detection yielding unreliable differences between control and experimental samples (**Fig. 3.5**).

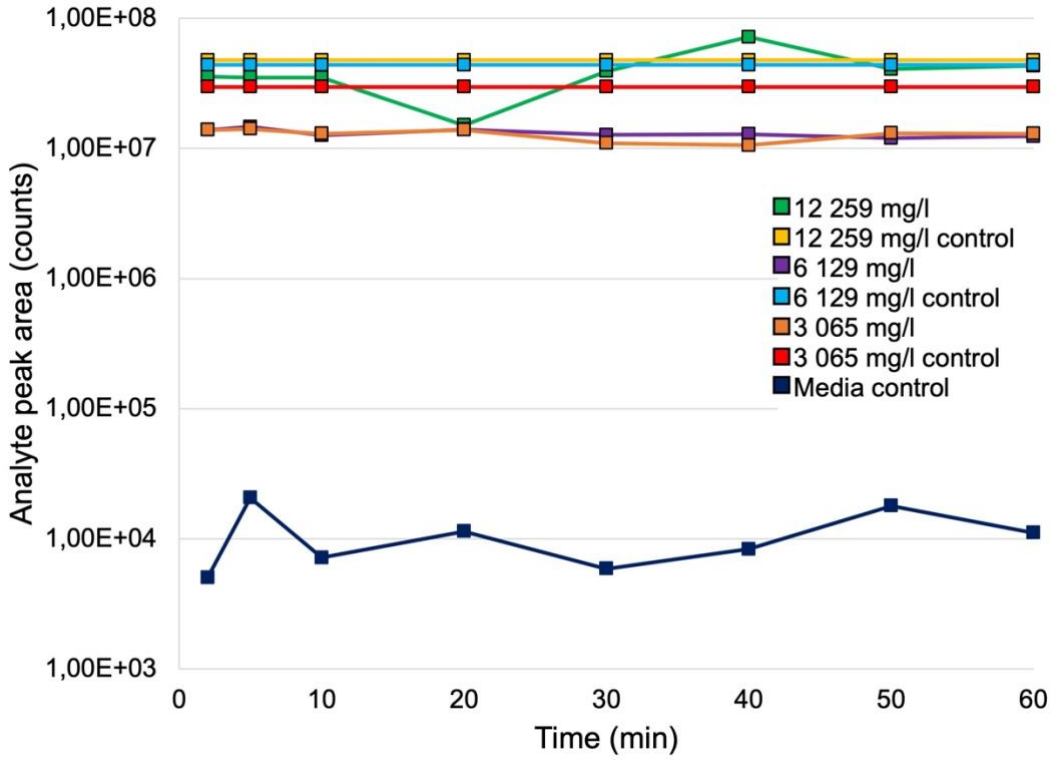
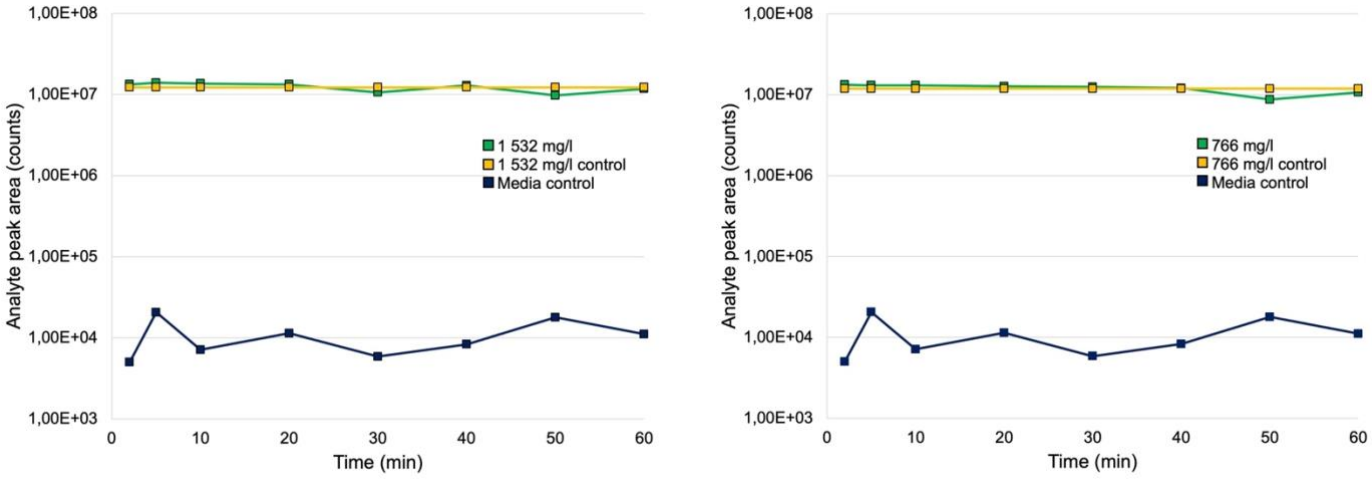


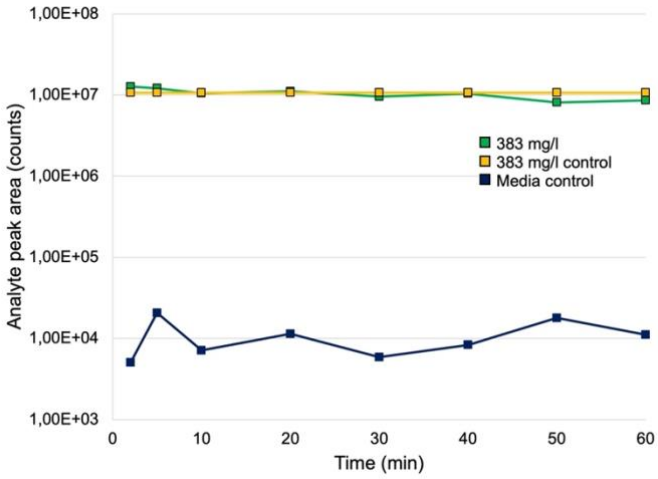
Figure 3.5: Relative quantification of chromatographic analyte peak areas of DDAC concentrations 12 259 mg/l, 6 129 mg/l and 3 065 mg/l.

Fig. 3.6 represent the DDAC concentrations (a) 1 532 mg/l, (b) 766 mg/l and (c) 383 mg/l. The data in **Fig. 3.6** exhibits no distinctive differences between the DDAC + HRI and DDAC dilution series analyte peak areas within the first 40 min of exposure. The *Serratia* sp. HRI was able to maximally reduce the analyte peak area of the 1 532 mg/l DDAC by 26% compared to the DDAC dilution control at t = 50 min (**Fig. 3.6** (a)). This reduction was lowered to 4% at t = 60

min, reiterating the detection inconsistency at high DDAC concentrations. *Serratia* sp. HRI could similarly reduce the DDAC level detected by a maximum amount of 37% at t = 50 min compared to the dilution series control concentration (**Fig. 3.6** (b)). This reduction was again lost at t = 60 min, where the DDAC detected was only lowered by 11%. The reductions in detection caused by HRI for the 383 mg/l DDAC followed more of a trend towards the end of the incubation period. At t = 50 min, the HRI + DDAC sample reduced 32% from the standard and at t = 60 min, the HRI + DDAC sample reduced 25% from the standard (**Fig. 3.6** (c)).



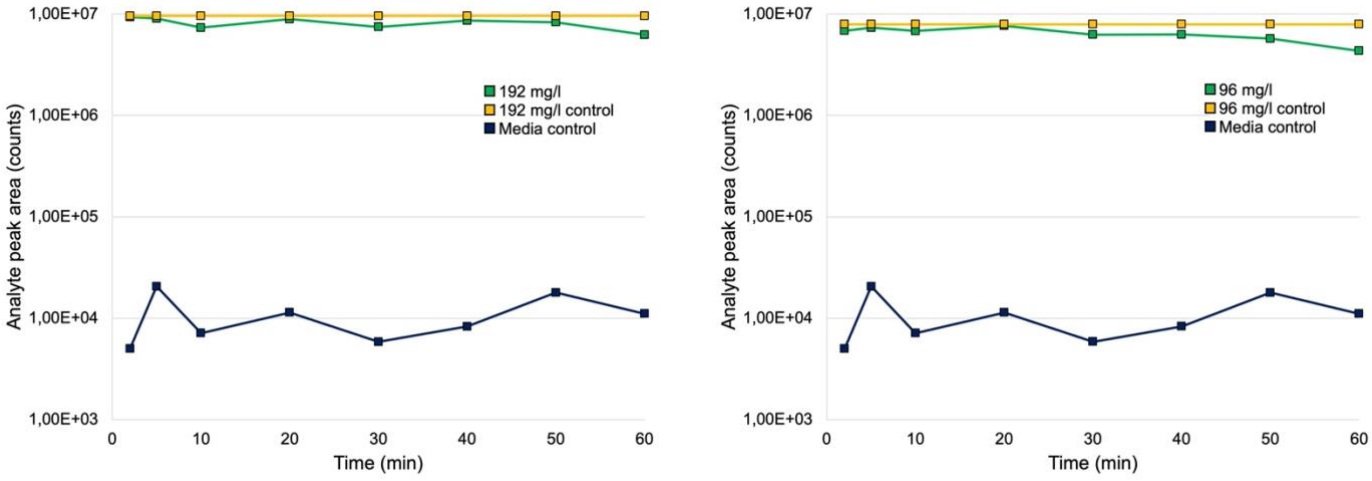
(a) (b)



(c)

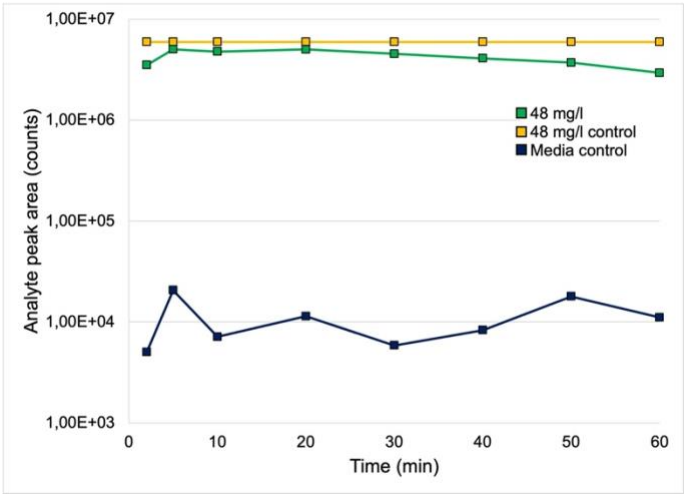
Figure 3.6: Relative quantification of chromatographic analyte peak areas of DDAC concentrations (a) 1 532 mg/l, (b) 766 mg/l and (c) 383 mg/l.

The analyte peak area changes recorded in **Fig. 3.7** (a), suggest that HRI was able to reduce the DDAC levels of the 192 mg/l DDAC at t = 60 min by 53% compared to the DDAC standard dilution series. The chromatographic peak areas of the 96 mg/l DDAC concentrations at each progressive time point from t = 20 min, showed a gradual decline in recorded counts compared to DDAC dilution series control (**Fig. 3.7** (b)). These reductions followed a trend of 27%, 26%, 38% and 83%, respectively, from time point t = 30 min. When considering the 48 mg/l DDAC concentration chromatographic peak areas, continuous reductions in the recorded counts per time point were exhibited (**Fig. 3.7** (c)). Reaching a maximal reduction of 102% from the DDAC standard dilution series at t = 60 min, which remained constant throughout.



(a)

(b)

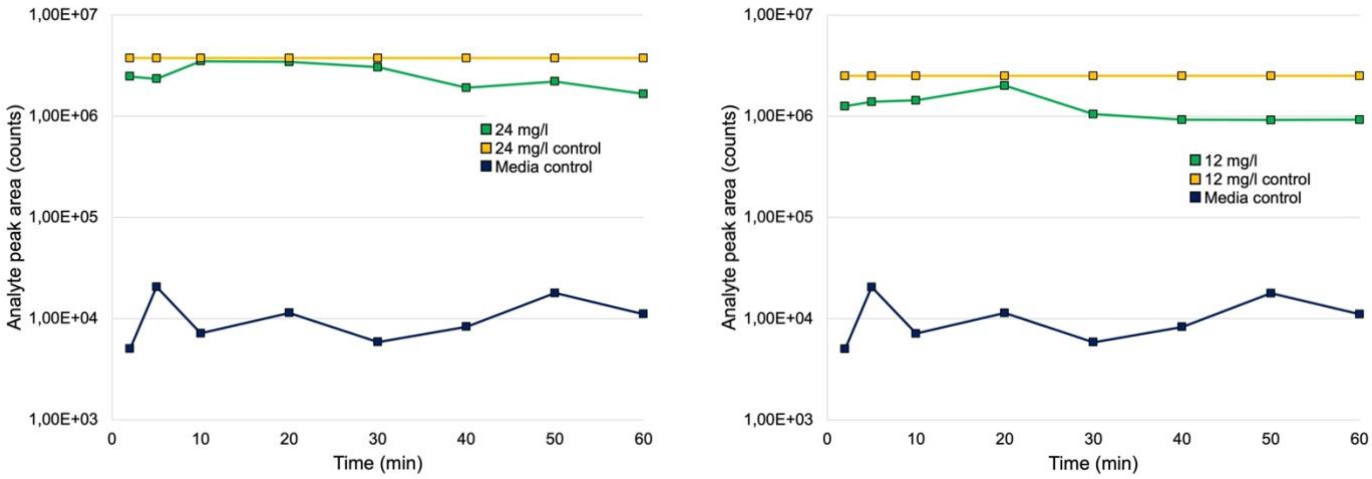


(c)

Figure 3.7: Relative quantification of chromatographic analyte peak areas of DDAC concentrations (a) 192 mg/l, (b) 96 mg/l and (c) 48 mg/l.

When considering the lowest concentration levels of DDAC tested, drastic reductions occurred compared to the DDAC standard dilution series. **Fig. 3.8** displays the reductions of 24 mg/l and 12 mg/l DDAC chromatographic analyte peak areas. The reductions present for the 24 mg/l DDAC + HRI samples compared to the DDAC dilution series decrease maximally at 126% (**Fig. 3.8** (a)). Furthermore, from t = 20 min there is increasing reduction percentages compared to the standard DDAC dilution series. The data exhibited from the 12 mg/l DDAC + HRI chromatographic analyte peak areas display drastic reductions compared to the DDAC

dilution series readings (**Fig. 3.8** (b)). From t = 30 min, there is a 139% reduction from the standard, culminating with a maximal reduction of 173% at t = 50 min.



(a) (b)

Figure 3.8: Relative quantification of chromatographic analyte peak areas of DDAC concentrations (a) 24 mg/l and (b) 12 mg/l.

3.4 Discussion

A robust method to appropriately evaluate antimicrobial treatment options *in vitro* is required to support tackling the threat of antimicrobial resistance. In this study, an *in vitro* time-kill assay was used to describe the relationship between the concentration of disinfectant, DDAC, and bacterial tolerance. Furthermore, assessing the role of efflux pump-mediated long-term disinfectant resistance by the addition of well-studied efflux inhibitor RSP.

The initial time-kill assay incorporated 4 hours of growth before adding the antimicrobial, this was to ensure the growth of the bacterial inoculum prior to testing. The assay time was limited to 6 hours. All the DDAC concentrations below 1 532 mg/l reached the upper limit of detection after time point t = 4 h (**Fig. 3.1**). The increases in the mean bacterial count for these DDAC concentrations suggests that the bacteria adapted to the environmental stress to allow survival

and promote proliferation. This is critical specifically for DDAC concentrations surpassing the recorded minimum inhibitory concentration (MIC) presented in **Fig. 3** in the published article in Chapter 2. The highest DDAC concentrations tested (12 259 mg/l and 6 035 mg/l) reduced the bacterial population to below the limit of detection during the first three hours of the experiment (**Fig. 3.1**). The bacterial population was able to recover to within the detection range providing evidence to substantiate that adaptation events lead to increased survivability.

The five DDAC concentrations (1 532 mg/l, 383 mg/l, 96 mg/l, 48 mg/l and 12 mg/l) selected for up scaling and further investigation, related to supra-inhibitory, inhibitory, and sub-inhibitory levels of disinfection (**Fig. 3.2**). These concentrations were catalogued based upon the MIC determination presented in **Fig. 3** in the published article from Chapter 2. Very minimal significant differences were recorded between the disinfectant and positive control mean bacterial counts. The bacterial counts of the 383 mg/l concentration exhibited the most significant differences at time points t = 3 h and 4 h. The lack of continuous significance throughout the analysis, proves the resistance capability of *Serratia* sp. HRI. Even though some of these concentrations are above the recorded MIC, the bacterial isolate was still capable of adaptation and cumulative population increases. HRI was able to eventually reach the upper limit of detection at the end of the time-kill assay for all five DDAC concentrations (**Fig. 3.2**).

To elucidate if this tolerance is resultant from efflux pumps, the efflux inhibitor (RSP) was added to the experimental design. RSP is a well-known EPI recognised to inhibit specific efflux pump superfamilies, such as the major facilitator , resistance-nodulation-division and ATP-binding cassette superfamilies (Braoudaki and Hilton 2005). The bacterial populations exposed to 1 532 mg/l and 383 mg/l DDAC concentrations experienced reductions in the mean bacterial counts after RSP was added at t = 3 h (**Fig. 3.3**). Interestingly, the comparison between the mean bacterial count with and without RSP addition showed that only 383 mg/l

was significant for the entire duration after t = 3 h. This suggests that efflux pumps are responsible, at least in part, for disinfectant tolerance recorded at this concentration. It is important to note that 383 mg/l DDAC is above the recorded MIC presented in **Fig. 3** in the published article from Chapter 2. This reinforces the idea that efflux pumps can contribute a major part to bacterial tolerance, even at supra-inhibitory concentrations.

The remainder of the disinfectant concentrations (96 mg/l, 48 mg/l and 12 mg/l) exhibited either slight reductions or no reductions in the mean bacterial count after RSP addition (**Fig. 3.3**). The recorded data also lacked any significant differences between the bacterial count with and without RSP addition. This suggests that at these DDAC concentrations, another tolerance mechanism is responsible for the survivability of the bacterial population. Certain intrinsic mechanisms of resistance could be responsible for the tolerance, such as membrane impermeability resulting from the double membrane of Gram-negative bacteria (Poole 2002). Although intrinsic resistance is highly plausible, investigating metabolism/degradation as a resistance mechanism specifically at lower DDAC concentrations proved to be an interesting prospect (Belter et al. 2022).

Bacteria can transport antimicrobial compounds and other substrates through the cell membrane using efflux pumps, decreasing the intracellular concentration to a non-lethal dosage (Piddock 2006). This transportation of antimicrobial compounds is generally considered to not interfere or alter with the structure of the molecules/chemicals that are being extruded. The molecule is transported through the membrane by structural changes in the efflux proteins resulting in changes in amino acids being displayed and their affinity for the chemical (Kumar and Varela 2012). A study involving the elemental analysis of *S. aureus* cells treated with DDAC found that trace amounts of chlorine could be detected (Jansen et al. 2013). These trace amounts were, however, too low to definitively conclude the presence or

absence of the compound. A similar DDAC treatment was conducted in the hopes to discover the unknown resistance mechanisms of *Serratia* sp. strain HRI.

LC-MS/MS was used to analyse DDAC spiked in bacterial growth samples in comparison to prepared DDAC serial dilution samples of the same concentration. LC-MS/MS has been widely used to analyse QACs in environmental water samples (Martínez Vidal et al. 2004). Liquid chromatography separates the inserted analytes into fragments and these fragments are detected by the mass spectrometer to allow possible determination of structure and quantification (Agilent Technologies 2001). Using this the level of QAC disinfectant in the growth media with and without bacterial presence was determined. Using relative quantification of the analyte peak areas of each DDAC spiked sample. This technique can be used to elucidate whether the bacteria can modify DDAC during the efflux process or not.

The detection limits of DDAC were determined using relative quantification of the chromatographic analyte peak areas of each DDAC concentration using LC-MS/MS. High DDAC concentrations were unreliably detected in DDAC spiked samples (**Fig. 3.5**). The relative quantification of 1 532 mg/l, 766 mg/l and 383 mg/l DDAC concentrations + HRI resulted in decreases from the control DDAC dilution series samples from t = 40 min (**Fig. 3.6**). There was between 25% - 37% reductions in quantified DDAC levels in comparison to the controls. Furthermore, the maximal reductions exhibited by 192 mg/l, 96 mg/l and 48 mg/l DDAC concentrations + HRI ranged from between 53% - 102% (**Fig. 3.7**). Finally, the lowest DDAC concentrations exhibited the highest percentage reductions with a maximal value of 173% (**Fig. 3.8**).

These decreases indicates that the original intact DDAC is no longer being detected in the same quantity suggesting there is less DDAC in samples containing HRI. A concentration dependent increase in percentage reduction of identified DDAC is evident. Thus, as DDAC

concentrations lower so the capacity of the bacteria to reduce the presence of intact DDAC increases (**Fig. 3.7** and **Fig. 3.8**). Additionally, the lack of efflux pump inhibition by RSP at the same DDAC concentrations where substantial decreases in intact DDAC detection occurred, suggests that some degradative mechanism could be responsible for the reduction in DDAC detection, and this warrants further investigation. The degradation of DDAC has been shown in bacteria, such as *Pseudomonas* (Nishihara et al. 2000). Thus, other less well-known mechanisms besides efflux pumps can occur in bacteria to facilitate disinfectant tolerance.

In conclusion, the potential of concentration-dependent efflux pump expression could be viable if the disinfectant molecules bind to specific repressors thereby, allowing the expression of efflux genes. As seen in the time-kill assays efflux pumps are involved in the long-term disinfectant resistance capability of *Serratia* sp. HRI shown by the significant decrease produced by RSP addition at specific DDAC concentrations (**Fig. 3.3**). However, the DDAC resistance in this isolate could also be derived from other mechanisms, such as degradation of DDAC. The relative quantification of the DDAC using the analyte peak areas lacks sensitivity when investigating high DDAC concentrations (12 259 mg/l, 6 129 mg/l and 3 065 mg/l) (**Fig. 3.5**). The reductions exhibited by the remainder of the tested DDAC concentrations suggest that some form of DDAC metabolism/degradation could be occurring (Belter et al. 2022). However, correlating the inability of RSP to inhibit efflux pump activity at the same DDAC concentrations where decreases in analyte peak areas was found is interesting and warrants further investigation. This study provides insight into how different resistance mechanisms can contribute to resistance to varying degrees depending on the concentration of the antibacterial agent.

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Chapter 4: General discussion and conclusions

4.1 General summary and conclusions

Bacterial resistance has increased to numerous antimicrobial agents in the last decade. The consistent presence and increase of antibiotic resistant microorganisms has been described as a global threat that could pave the way to a post-antibiotic era (Roca et al. 2015). Disinfection protocols and good biosecurity practices will be essential to control bacterial populations when antibiotics fail (Bragg et al. 2018). Quaternary ammonium compounds (QACs) are common disinfectants used in a wide range of products ranging from industrial-grade disinfectants to household cleaning agents (Paulson 2002).

The occurrence of disinfectant resistance is therefore very alarming. What is even more alarming is that the emergence of disinfectant resistance is occurring in conjunction with the promiscuous antibiotic resistance (Kampf 2018, 2019; Kim et al. 2018). Disinfectant resistance development occurs through these agents' misuse and abuse. Particularly, when inadequate application and management of these compounds occur in relevant environments where clinically significant isolates are present, such as in the hospital setting (Campoccia et al. 2010). The inadequate and misuse of disinfectants have been linked to antibiotic resistance emergence without exposure to antibiotics (Roca et al. 2015; Kim et al. 2018). This co-resistance capability is often due to the presence and spread of mobile genetic elements harbouring genes that confer multiple antimicrobial resistance mechanisms (Chapman 2003). Cross-resistance can be achieved through various antimicrobials sharing common modes of action targeting the same site, resulting in the recruitment of common resistance mechanisms (Chapman 2003). Multidrug efflux pumps are often the source of co-resistance in bacterial isolates conferring tolerance capabilities to both antibiotics and disinfectants (Tezel and Pavlostathis 2012). Horizontal gene transfer between different bacteria in an environment allows the acquisition of resistance genes through plasmid transfers (Hegstad et al. 2010).

Specifically, QAC resistance can be encoded by plasmid-mediated QAC-specific efflux proteins and multidrug efflux proteins (McDonnell and Russell 1999). Understanding whether efflux pumps play a major role in QAC resistance will be crucial in the fight against multidrug-resistant bacteria.

Serratia sp. HRI, possessing high disinfectant resistance characteristics, was isolated from a diluted solution of didecyl dimethylammonium chloride (DDAC)-based disinfectant, possessing high disinfectant resistance characteristics. This isolate provides a suitable platform to investigate the known mechanisms of disinfectant resistance with the potential to associate poorly understood mechanisms. By studying this resistant isolate, the following research questions were answered:

1) Do efflux pumps contribute to the high level of disinfectant resistance present in *Serratia* sp. HRI during short-term disinfection?
2) What are the responsible mechanisms conferring resistance present in *Serratia* sp. HRI during long-term disinfection?

Efflux pumps can be one of the most rapid and effective resistance mechanisms for bacteria to respond to the stress of antibacterial agents (Neuberger et al. 2018). Therefore, to answer these research questions and fulfil the aims of the study, the first objective was to determine if resistance efflux pumps are present in *Serratia* sp. HRI. Thereafter, the activity of the efflux pumps was assessed using known efflux pump inhibitors (EPIs) of specific efflux superfamilies. The long-term resistance capability of *Serratia* sp. HRI was assessed using time-kill assays. Finally, the investigation of other resistance mechanisms involving metabolic activity or degradation was conducted using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

In Chapter 2, efflux pump superfamilies were identified in the *Serratia* sp. HRI and closest related type strain *Serratia marcescens* subsp. *marcescens* ATCC 13880 presented in **Fig. 1** in the published article from Chapter 2. Interestingly, the total number of efflux pumps and the number of resistance efflux pumps in the ATCC strain were greater than the HRI isolate as presented in **Fig. 2** in the published article from Chapter 2. Of the total predicted efflux pumps, very few were known to confer resistance capabilities. Several QAC-specific efflux pumps were, however, predicted in both the *Serratia* strains from the small multidrug resistance (SMR) subfamily and the major facilitator superfamily (MFS) listed in **Table S1** and **Table S2** in the published article from Chapter 2. Additionally, many multidrug-resistant efflux pumps from most of the five superfamilies were identified in both strains listed in **Table S1** and **Table S2** in the published article from Chapter 2.

The minimum inhibitory concentration (MIC) testing of three disinfectants confirmed the resistance capabilities of the HRI isolate in comparison to the ATCC strain as presented in **Fig. 3** in the published article from Chapter 2. Furthermore, the supplementation of reserpine (RSP), one of the EPIs tested, produced a significant reduction in the activity of specific efflux pumps in both strains as presented in **Fig. 4** and **Fig. 5** in the published article from Chapter 2. All the tested disinfectant MIC values were statically significantly decreased after the addition of RSP. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), the other EPI tested, produced mixed results depending on the disinfectant and strain tested as presented in **Fig. 4** and **Fig. 5** in the published article from Chapter 2. The supplementation of CCCP produced statistically significant increases and decreases in the susceptibility of the bacterial strains. The paradoxical decrease in susceptibility to certain disinfectants was concluded to be from the Eagle effect. The Eagle effect is described as the paradoxical reduction in the killing capacity of antibiotic concentrations higher than the optimal bactericidal concentration against microorganisms (Prasetyoputri et al. 2019). The Eagle effect has not been described as a phenomenon that occurs during disinfection, however, the EPI-treated susceptibility results

presented here fit this trend. Future work can include gene knockout mutation experiments, specifically targeting efflux pump genes predicted in resistance superfamilies inhibited by RSP. The work presented in this chapter confirms that resistance efflux pumps are present in *Serratia* sp. HRI. Additionally, those specific superfamilies are involved in conferring disinfectant resistance to QACs and HyperCide®.

In Chapter 3, the long-term resistance capability of *Serratia* sp. HRI was assessed by time-kill assays. The time-kill assays provided insight into how the HRI isolate tolerates various concentrations of DDAC. The HRI isolate showed varying degrees of tolerance capabilities for all the concentrations of DDAC tested. At the highest DDAC concentrations tested, the HRI isolate displayed remarkable tolerance to disinfection (**Fig. 3.1**). The highest DDAC concentrations tested (12 259 mg/l and 6 129 mg/l) decreased the mean bacterial count (CFU/ml) of HRI below the limit of detection within the first 3 hours of the experiment. However, the strain recruited a mechanism or mechanisms of resistance during this period to nullify/prevent the activity of DDAC promoting bacterial proliferation. From the data in the publish article from Chapter 2, it was concluded that efflux pumps are involved in DDAC resistance as presented in **Fig. 4**. The possibility of efflux pump activity in this environmental capacity of long-term disinfection was determined by adding RSP. From the five selected DDAC concentrations (1 532 mg/l, 383 mg/l, 96 mg/l, 48 mg/l and 12 mg/l) used in the up-scaled flask investigations, the RSP addition only affected 383 mg/l concentration to induce statistically significant differences from the uninhibited time-kill assay (**Fig. 3.3**). This suggests that the HRI isolate utilises efflux pumps to confer tolerance to DDAC at this concentration. A possibility could be that at specific disinfectant concentration thresholds particular resistance mechanisms are recruited.

The addition of RSP had negligible effects on the DDAC concentration closest to the MIC (96 mg/l) as determined in the published article from Chapter 2 presented in **Fig. 3**. This can be

expected as the bacteria during the time-kill assay have had ample time to adjust to the environmental stress in comparison to the brief time during the MIC determination (**Fig. 3.3**). When RSP was added with disinfectant concentrations below the recorded MIC (48 mg/l and 12 mg/l), minimal effects were exhibited, and no significant differences were recorded (**Fig. 3.3**). The lack of effective inhibition suggests that other mechanisms of resistance besides efflux are involved in conferring tolerance in diluted disinfectant concentrations.

Evaluating the degradation or metabolic capabilities of the HRI isolate was conducted using LC-MS/MS. The same range of DDAC concentrations were investigated as those in the time-kill assays. Comparative analysis of the control DDAC dilution series and the experimental DDAC dilution series with the addition of the HRI strain showed that HRI has the potential for some metabolic or degradation capabilities (**Fig. 3.7** and **Fig. 3.8**). The relative quantification of the analyte peak areas of the DDAC chromatograms revealed that at lower DDAC concentrations sharp reductions in the recorded peak areas occurred (**Fig. 3.7** and **Fig. 3.8**). The higher concentrations of DDAC were above the accurate limits of detection for the LC-MS/MS apparatus, preventing definitive conclusions to be drawn from this data (**Fig. 3.5**).

The correlation between the ineffective inhibition of RSP and the decrease in chromatogram analyte peak areas of less concentrated DDAC samples indicates that transportation by efflux proteins is not involved in altering the structure of the DDAC. If efflux proteins were involved in structural alteration of DDAC, they would need to be in a functional capacity as evidenced by the reduction in mean bacterial count after RSP addition (**Fig. 3.3**). Therefore, another process, such as metabolism or degradation, could be responsible for the lower DDAC quantified for the 96 mg/l, 48 mg/l and 12 mg/l concentrations (**Fig. 3.7** (b), (c) and **Fig 3.8** (b)). Future work can include the identification and knockouts of known QAC degradative or metabolic genes. Additionally, when considering how disinfection occurs in real-world

scenarios, transcriptomic or RNA sequencing studies can be conducted to determine regulatory and expression changes during disinfection at various concentration thresholds.

The answers to the research questions stated at the beginning of this work are summarised below. *Serratia* sp. HRI has high levels of disinfectant resistance toward QACs due to efflux pump activity. This efflux-mediated resistance seems to be representative of short- and long-term disinfection. However, efflux is not the only mechanism in this isolate's repertoire and metabolism, or degradation of QACs could also be an active part in providing tolerance. The hypothesis posed in the first chapter of this thesis can be accepted as this work provides evidence that *Serratia* sp. HRI is highly resistant to disinfectants conferred by efflux-mediated resistance mechanisms.

The work completed in this thesis has shed light on the intricacies of disinfection and disinfectant resistance. This work has highlighted the role of efflux pumps as vital parts of disinfectant tolerance in susceptible and resistant isolates. Additionally, this work has provided more insight into the limited knowledge of bacterial metabolism or degradation of disinfectants. This work reinforces the idea that a single resistance mechanism cannot protect a single antimicrobial agent at all concentrations of that agent. Combinations of resistance mechanisms provide overall protection of bacterial populations against antimicrobial threats in the most energy-efficient manner available. This thesis includes one research paper (Susceptibility Tests and Predictions of Transporter Profile in *Serratia* Species) published in an open-access, peer-reviewed, accredited journal, "**Microorganisms"**, impact factor 4.926.

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