

DISSERTATION SUBMISSION

Title:

Molecular insights into disinfectant resistance of a highly resistant *Serratia* species.

Full names and surname:

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Submission date:

December 2024

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Submitted in fulfilment of the requirements of a doctoral degree with specialisation in Microbiology in the department of Microbiology and Biochemistry in the faculty of Natural and Agricultural Science at the University of the Free State.

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I, Samantha J. Mc Carlie, declare that the thesis (or interrelated, publishable manuscripts/published articles or mini-thesis) that I herewith submit for the Doctoral Degree 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.



01-12-2024

Department of Microbiology & Biochemistry

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Abstract

Antimicrobial resistance (AMR) is a global crisis, and with fewer antibiotics entering the market, disinfectant resistance poses an emerging threat. This dissertation investigates the mechanisms of high Quaternary Ammonium Compound (QAC) resistance in *Serratia* sp. HRI. This work identified multiple resistance mechanisms, focusing on the stress response as a key regulator. In Chapter 1, a signature transcriptomic profile of benzalkonium chloride (BC) was generated by reviewing available literature on the transcriptomic response of several bacteria to BC. The transcriptomic response of bacteria to BC exposed minimal correlation with known QAC resistance mechanisms, suggesting novel pathways. Chapter 2 revealed a hormetic effect in which sub-MIC levels of disinfectants stimulated bacterial growth, potentially increasing infection risks. Chapter 3 observed no biofilm formation but identified outer membrane vesicles (OMVs) in the resistant isolate, suggesting OMVs contribute to QAC resistance, potentially acting as a barrier similar to their role in antibiotic resistance. Further analysis in Chapters 4 and 5, revealed up-regulation of an uncharacterised MFS pump and polyamine synthesis pathways, both involved in disinfectant resistance through efflux mechanisms. Differential gene expression indicated that efflux pumps and stress response systems act in tandem to confer resistance. Susceptibility tests confirmed the role of the stress response in increasing QAC resistance in *Serratia* sp. HRI. Overall, this research highlights the complex, multifaceted nature of disinfectant resistance, proposing that the stress response regulates various resistance mechanisms, including efflux pumps and OMVs. This study offers new insights into disinfectant resistance, suggesting that future strategies should focus on understanding and mitigating the stress response to preserve the efficacy of disinfectants.

List of Abbreviations

AMR: Antimicrobial resistance

BC/BAC: Benzalkonium chloride

DDAC: Didecyldimethylammonium chloride

DGE: Differential Gene Expression

dNTP: Deoxynucleoside triphosphate

EPA: United States Environmental Protection Agency

MIC: Minimal Inhibitory Concentration

NCBI: The National Centre for Biotechnology Information

OMV: Outer Membrane Vesicle

ORF: Open Reading Frame

QAC: Quaternary Ammonium Compound

RAST: Rapid Annotation using Subsystem Technology (Automated service tool)

UMFS: Uncharacterised Major Facilitator Superfamily

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Chapter 1: Literature Review

1.1 Introduction to the current study

The SARS-CoV-2 pandemic highlighted our reliance on disinfectants and sanitisers as crucial components for biosecurity and public health (Ma *et al.* 2020; Meade, Slattery and Garvey 2021). As we are currently in an antibiotic resistance crisis with a lack of alternative treatment options, we must prioritise disease prevention over treatment as more antibiotic-resistant microorganisms emerge (Bragg *et al.* 2018). Disease prevention is the focus of biosecurity, which can be defined as a set of measures to prevent the establishment or spread of harmful microorganisms (Perkins *et al.* 2018; Hulme 2020; Ma *et al.* 2020). Biosecurity relies heavily on effective disinfectants, antiseptics and sanitisers (biocides), which often share the same active ingredients and thus can be studied collectively (Gerba 2015; Liu *et al.* 2017).

Disinfectants are used in common household cleaning products and in industries such as the food and beverage industry to maintain hygiene and safety standards and prevent food-borne diseases (Pereira and Tagkopoulos 2019). These products are used extensively in the agricultural sector in infection control and to meet strict export safety standards and, by extension, safeguard food security (Gibbens *et al.* 2001; Postma *et al.* 2016). Disinfectant products are also used in the pharmaceutical, cosmetic and other industries to maintain manufacturing and product quality control (Gandhi *et al.* 1993; Pereira and Tagkopoulos 2019; Jones and Joshi 2021).

Many active ingredients are used in biocides, such as chlorine, chlorhexidine, povidone iodine, alcohol, sodium hypochlorite, phenols, hydrogen peroxide, Quaternary ammonium compounds (QACs) and glutaraldehyde (EPA 2021). When considering the list generated by the United States Environmental Protection Agency (EPA), as depicted in Figure 1, QACs make up the greatest proportion of recommended disinfectants (38%) approved for use against SARS-CoV-2 (EPA 2021). Within this

majority, benzalkonium chloride (BC/BAC), a first-generation QAC, is present in 92% of the approved QAC products (EPA 2021).

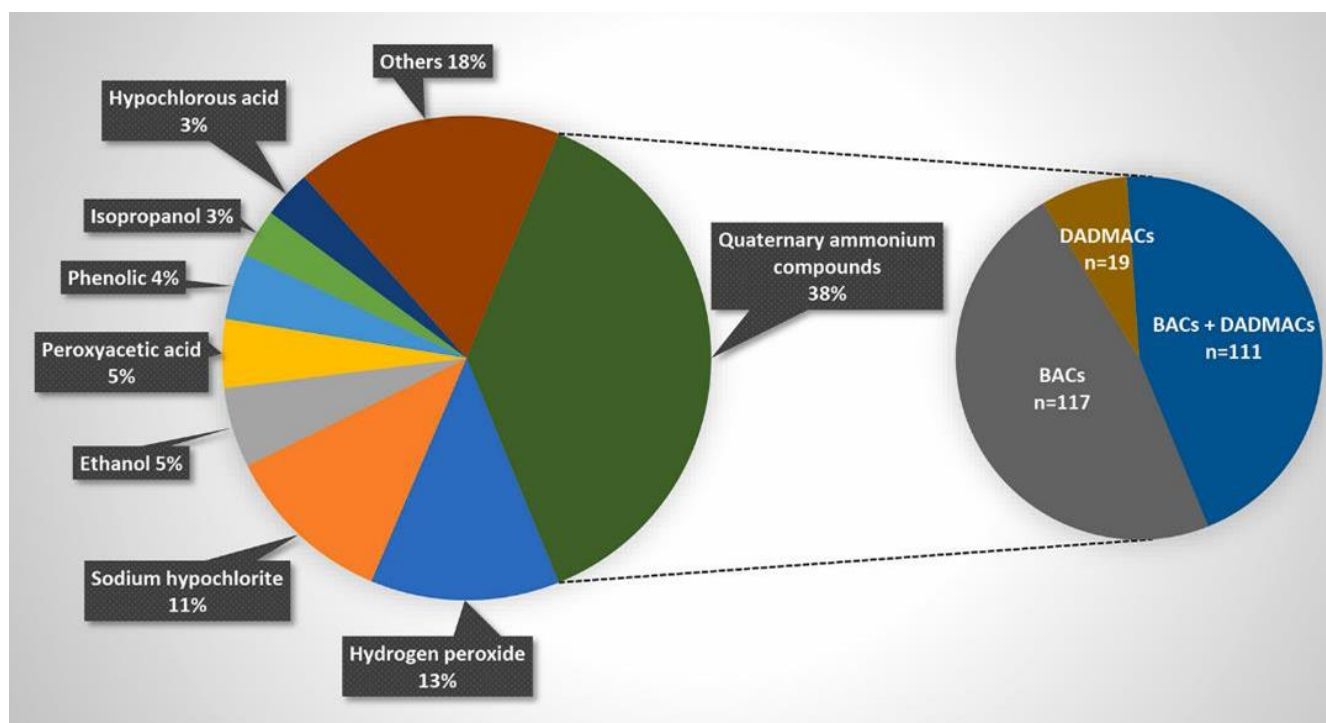


Figure 1.1. List N: Active ingredients in EPA-approved disinfectants for use against SARS-CoV-2 (EPA 021).

Disinfectants and antiseptics played a crucial role in infection control during the pandemic and, currently, are the only viable alternative to antibiotics in controlling microbial growth and combating disease (Allen *et al.* 2014; Bragg *et al.* 2018; Rello, Parisella and Perez 2019). However, microbial resistance to biocides is now emerging at an alarming rate, accelerated by their extensive and misuse during the COVID-19 pandemic (Rezasoltani *et al.* 2020; Lai *et al.* 2021; Rizvi and Ahammad 2022; Petrakis *et al.* 2023). Furthermore, recent research has shown that resistance to disinfectants and antibiotics can evolve simultaneously through shared mechanisms which reduce susceptibility to a wide range of antimicrobials (Kim *et al.* 2018b; Merchel Piovesan Pereira, Wang and Tagkopoulos 2021). Kim and co-workers (2018) found that exposure to benzalkonium chloride can promote the

development of antibiotic resistance by co-selecting for antibiotic resistant microorganisms. Therefore, research into disinfectant resistance should be prioritised in the study of antimicrobial resistance.

Although resistance to disinfectants is a relatively novel field when compared to antibiotic resistance, several mechanisms of disinfectant resistance have been elucidated (McDonnell and Russell 1999; Chapman 2003; Kim *et al.* 2018a; Mc Carlie, Boucher and Bragg 2020; Milani *et al.* 2021). Mechanisms of disinfectant resistance include efflux pumps, target alteration, cell membrane changes, biofilm formation, and disinfectant inactivation and metabolism (as reviewed by Chapman, 2003). Many of these mechanisms can be traced back to individual resistance genes (*qacA*, *smr*) (Longtin, Seah and Siebert 2011) or operons (*mexCD-oprJ*) (Fraud *et al.* 2008), which can be located on the chromosome (*qacH*) (Müller *et al.* 2013) or on extrachromosomal elements such as plasmids (*qacJ*) (Bjorland *et al.* 2003). The significant advances in sequencing and bioinformatics analyses have led to the characterisation of many molecular mechanisms of antimicrobial resistance (Van Camp, Haslam and Porollo 2020). The frontier of sequencing and bioinformatics stretches further yearly with greater accuracy and depth of sequencing paired with more robust bioinformatics pipelines (Her and Wu 2018; Subramanian *et al.* 2020; Olson *et al.* 2023). Gene and protein annotation databases are expanding daily with increased user input and more reliable annotations (Tedim *et al.* 2024; Wattam *et al.* 2024). These tools are invaluable in the fight against antimicrobial resistance and can be used to confirm what is known about resistance and elucidate novel resistance mechanisms (Her and Wu 2018; Van Camp, Haslam and Porollo 2020; Wattam *et al.* 2024).

Serratia sp. HRI is an isolate with high QAC resistance capabilities (Mc Carlie *et al.*, 2020b) and therefore is an attractive candidate in the study of disinfectant resistance. In this work, *Serratia* sp. HRI will be studied with the closest related type strain, *Serratia marcescens* subsp. *marcescens* ATCC 13880 (Mc Carlie *et al.* 2020). Whole genome sequencing in previous work revealed 97 antimicrobial resistance genes within the genome of *Serratia* sp. HRI (Mc Carlie *et al.* 2020). However, the presence of these genes alone does not guarantee that they are playing a role in this isolate's high levels of

resistance. Therefore, the following research questions will be addressed in the study: (1) What mechanisms are responsible for the high levels of QAC resistance in *Serratia* sp. HRI? (2) Does the differential expression of resistance genes play a role in the disinfectant resistance of *Serratia* sp. HRI? (3) Are there multiple mechanisms of resistance acting simultaneously to confer the resistance phenotype in *Serratia* sp. HRI?

1.2 Aims and objectives

This dissertation aims to identify mechanisms involved in high levels of QAC resistance in *Serratia* sp. HRI. The hypothesis for this work is that for *Serratia* sp. HRI there exists a combination of mechanisms, both known and well-characterised and perhaps novel, uncharacterised mechanisms as well.

To uncover the resistance mechanisms of this isolate, this research focused on how the bacterium responds to treatment with QAC disinfectants compared to the closest related type strain, *Serratia marcescens* ATCC 13880. By learning more about the response of this bacterium to an attack by QAC compounds, it is possible to elucidate the mechanisms behind the resistance phenotype. The survival mechanisms of *Serratia* sp. HRI distinguishes this isolate and allows a unique opportunity to study QAC resistance.

To elucidate which survival mechanisms, confer the resistant phenotype of *Serratia* sp. HRI both phenotypic and molecular analysis was conducted in a holistic approach. The first objective was to determine if cell morphology and biofilm formation of *Serratia* sp. HRI were affected by QAC-treatment at sub-MIC levels compared to the type strain. This work is presented in Chapter 3 as an article that is currently in the second review stage. The second objective of this study marked the move towards molecular analysis in the form of RNA-Seq, to screen for novel disinfectant resistance genes by differential gene expression in *Serratia* sp. HRI when exposed to sub-MIC levels of benzalkonium chloride. Treated samples were compared to untreated samples and gene lists of possible benzalkonium chloride resistance genes were generated. This work is presented in Chapter 4 as a published article.

Finally, the third objective was to build on the previous work by analysing differential gene expression of *Serratia* sp. HRI during treatment with two additional QAC disinfectants (DDAC and Viurkill™). However, the focus changed from screening for resistance genes to assessing differential gene expression of metabolic systems and their impact on QAC resistance and susceptibility. This work is presented in Chapter 5 as a published article.

The literature review section of this thesis has been published and is entitled “Transcriptomic signature of bacteria exposed to benzalkonium chloride” in the journal *Research in Microbiology*. This is a peer-reviewed and accredited scientific journal. As the first author, I researched the topic, wrote, and compiled the article. The co-authors of this article are two promoters for this dissertation, namely, Prof. R. R. Bragg and Dr C. E. Boucher-van Jaarsveld, who were integral to the initial conceptualisation and review and editing of the paper. Additional co-authors are Dr. L. du Preez and Dr. J Castillo Hernandez, who assisted in the methodology design, results analysis, and final editing and review. This published literature review is presented as the complete literature review of this thesis.



Review

Transcriptomic signature of bacteria exposed to benzalkonium chloride

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ARTICLE INFO

Article history:

Received 8 June 2023

Accepted 1 November 2023

Keywords:

Antimicrobial resistance

RNA-Seq

Quaternary ammonium compounds

ABSTRACT

The COVID-19 pandemic has highlighted our reliance on biocides, the increasing prevalence of resistance to biocides is a risk to public health. Bacterial exposure to the biocide, benzalkonium chloride (BAC), resulted in a unique transcriptomic profile, characterised by both a short and long-term response. Differential gene expression was observed in four main areas: motility, membrane composition, proteostasis, and the stress response. A metabolism shift to protect the proteome and the stress response were prioritised suggesting these are main resistance mechanisms. Whereas “well-established” mechanisms, such as biofilm formation, were not found to be differentially expressed after exposure to BAC.

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1. Introduction

Antibiotic resistance is a well-known problem with far-reaching effects in the medical and agricultural industries. However, this is a foreshadowing of a potentially larger issue which is microbial resistance to biocides. Biocides are substances with one or more active ingredients with microbicidal effects used to control and prevent the establishment of a microbial population. Biocides include disinfectants, antiseptics and sanitisers. Disinfectants and antiseptics are used extensively in medical environments to prevent nosocomial infections and protect against disease [1]. These compounds are also used in large quantities in the agricultural and food industry to maintain food safety and disease control. Disinfectants are crucial to protect food security and international exports as they are used to uphold essential hygiene standards in food processing as reviewed by Jones and Joshi [2]. Biocides are even used in pharmaceutical and cosmetic products as preservatives [3]. The COVID-19 pandemic has highlighted our reliance on disinfectants and sanitisers [4e6] and this, together with their widespread use, makes it troubling that resistance to disinfectants is emerging at an accelerated rate [7,8].

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Benzalkonium chloride (BAC) is a first-generation Quaternary Ammonium Compound (QAC) disinfectant [9]. QAC disinfectants are popular worldwide in many industries due to their efficacy, low toxicity and broad range of target organisms [3]. As of March 2021, the EPA listed 535 approved disinfectant products to be used for COVID-19. Of these 535 products, the largest proportion, at 38 %, included those with QAC compounds as the active ingredient: with a total of 228 products using BAC as an active ingredient (List and statistics available at: <https://cfpub.epa.gov/giwiz/disinfectants/index.cfm>). Several new generations of QACs have been developed, however, the greatest amount of research has focused on BAC with significantly fewer studies on any other QAC compound. In addition, not enough literature exists yet for other biocides to produce a meaningful review. For these reasons, this review will focus on BAC, specifically the mode of action, what resistance mechanisms are known and the insight transcriptomics provides about the bacterial response to BAC. This information can be used as a starting point for similar work on more recently developed QAC disinfectants, such as didecyldimethylammonium chloride (DDAC).

The mode of action of a biocide can provide insight into how it can be counteracted and thus hint at the possible evolutionary path towards microbial resistance [9]. New sequencing technology and more accessible bioinformatics programs have allowed for great advances in the study of biocide resistance. Transcriptomics in particular is a powerful tool that provides insight into the cell-wide

effects of antimicrobials [10,11]. Instead of studying a certain gene, protein or system, transcriptomics paints an overall picture of what occurs inside the cell at a particular moment. This allows for the creation of transcriptomic profiles for each antimicrobial as we can observe which systems are affected when a microorganism is exposed to an antimicrobial. In addition, this technology can go a step further and generate a related resistance profile for each antimicrobial based on the response of resistant microorganisms. If the resistance mechanisms for an antimicrobial are understood, this is the first step to counter the emergence of resistant populations and can be used to direct the design of new antimicrobials. Alternatively, this information on the mechanisms of antimicrobials can allow us to make better use of current products, by either using different combinations or rotating antimicrobials, both of which will aid in delaying the emergence of resistance.

This paper aims to review the bacterial response to BAC, depicted by differential expression and correlate the resultant differential gene expression to the mode of action of BAC and resistance mechanisms against BAC. To the best of the authors knowledge, this is the first paper to review the impact of any QAC-based biocide on differential gene expression and is the first to generate a transcriptomic profile for benzalkonium chloride.

2. Mode of action of benzalkonium chloride

Benzalkonium chloride, like other QAC disinfectants, are characterised by long alkyl chains and a positively charged ammonium group [12]. QAC activity is based on the length of the hydrophobic tail, type of side chain and number of cationic residues of the QAC molecule [13]. Briefly, when BAC is introduced into the environment it is attracted to the bacterial cell wall by the electrostatic nature of negatively charged lipids [9,14]. The hydrophobic tail of BAC integrates into the bacterial cell membrane and causes a lateral diffusion of lipids destabilising the cell membrane, leading to disintegration [9,12]. Disruption of the cell membrane results in a leakage of intracellular contents and nucleic acid and protein degradation [9,12]. Autolytic enzymes lyse the cell wall and result in cell death [12,15]. Thus, the mode of action of BAC and other QAC-based disinfectants is focused on targeting the cell wall and microbicidal effects are brought about by membrane disruption and leakage of cell contents.

3. Mechanisms of resistance to BAC

There are no widely accepted definitions for the terms “tolerance” and “resistance” to biocides. For antibiotics, microbial resistance is defined as an inherited ability to survive at a high concentration of an antibiotic, this is defined without a specific duration of treatment and depends on the minimum inhibitory concentration (MIC) for a specific antibiotic [16]. Tolerance is defined as the survival of a microorganism, after treatment by a high concentration of an antibiotic, without a change in the MIC, that would otherwise be lethal [17,18]. Tolerance often occurs by slowing down an essential bacterial process such as cell growth which confers tolerance to β -lactam antibiotics which rely on an active cell wall assembly to bring about antibacterial effects [19]. These terms have not been well-defined for biocides as the microbicidal effect for biocides depends on several factors, including organic load, contact time, temperature, pH and concentration [20]. Altogether, these factors can all greatly influence the MIC of a biocide and therefore the definitions used for antibiotics cannot be applied to biocides [21]. Cerf and co-workers [16]

suggest that for biocides and disinfection, “resistance” be used when a microbicidal or killing effect is being studied, and “tolerance” be used when adaption to an inhibitory effect is seen. Although resistance to biocides is a relatively novel field, it is widely accepted that the most common mechanism of biocide resistance is efflux pumps systems. Efflux pumps reduce the intracellular concentration of the biocide and therefore limit intracellular damage, these efflux systems can be specific for biocides such as the *qac* and *smr* families of efflux pumps [22-24], or can function as multidrug resistant pumps as reviewed by Chitsaz and Brown [25]. Adaptions to the cell envelope is another well-characterised biocide resistance mechanism, adaptions include altering the charge of the cell membrane, modification to lipopolysaccharides and lipid A residues to hinder antimicrobial adsorption and lower permeability of the outer membrane [26,27]. In addition, the composition of the outer membrane can change to include more efflux pump proteins and less porins which biocides use for entry into the cell [27]. An increased mutation rate can also give rise to resistance as biocide targets can be altered. Resistance to the biocide triclosan is brought about by mutations in its target, the *fabI* gene [28]. However, this mechanism is not common as most biocides have multiple non-specific targets and therefore even after substantial mutations the target molecule may still be vulnerable [9].

Biofilm formation is also a well-known survival mechanism to reduce susceptibility to antimicrobials [29-32]. However due to their surfactant properties quaternary ammonium compound disinfectants, such as BAC, have been found to inhibit filamentation, the first stage of biofilm formation, in *Candida albicans* [15]. More research is being done on the degradation and metabolism of biocides by bacterial populations and it seems this could be an effective mechanism of resistance as biocides are deactivated and then metabolised by bacteria [33-35]. Lastly, the acquisition of resistance determinants can bring about resistance to biocides through horizontal gene transfer within a microbial population [36-38]. Multiple resistance genes can be transferred horizontally on the same mobile element resulting in co-resistance to a variety of biocides and antibiotics within a single microorganism [39].

Cross-resistance between antimicrobials is often seen in isolates with common resistance mechanisms, such as efflux pumps or changes in cell membrane charge that are effective against both biocides and antibiotics [26,27,40]. This gives rise to multidrug resistant isolates that are resistant to a broad range of antimicrobials [41,42]. Due to common mechanisms, resistance can develop without exposure or selective pressure; exposure to benzalkonium chloride promotes resistance to both BAC and antibiotics even without prior antibiotic exposure because of shared resistance mechanisms [43]. The implication of this work is that the development of biocide resistance can result in simultaneous development of resistance to a broad range of antimicrobials; a major cause for concern.

4. Sequencing technology

Recent advances in sequencing technology and bioinformatics programs have allowed researchers to get a glimpse of what occurs in real-time within a bacterial cell. Genomics in the form of whole genome sequencing and genome annotation can be used to uncover which resistance genes are present within a microorganism

[44]. This can be used to generate a genotypic resistance profile and predict reduced susceptibility of an isolate to certain antimicrobials [45]. However, the resistance determinants need to be known and compared to a database to be identified. Therefore, it can be difficult

to identify novel mechanisms of resistance with this technology. Once identified and characterised, whole genome sequencing can reveal which resistance genes are present within a genome, but not if these genes are active or playing any role in the resistance phenotype. Although limited, whole genome sequencing is a vital tool to study reduced susceptibility to disinfectants and other biocides. Subedi and co-workers [44] used whole genome sequencing to study clinical ocular isolates of *Pseudomonas aeruginosa*. This study showed that an increase in keratitis infections can be associated with disinfectant resistant strains. Whole genome sequencing was used to determine which biocide resistance genes were present in these isolates and susceptibility profiles were generated. Furthermore, the presence or absence of antibiotic and biocide resistance genes were compared between isolates from different regions and countries, adding to the work on surveillance of resistance determinants [44]. This research is vital for the surveillance of resistance genes and can be used to direct future treatment; by knowing which resistance genes are present in a population, a change in the type of disinfectant used in clinical settings or industry can be recommended.

Genomics can also be used to track the prevalence and spread of resistance determinants between industries. Gelbicova and coworkers [46] sequenced 270 *Listeria monocytogenes* isolates from clinical settings, the food industry and environmental isolates. This study found that genes encoding resistance to benzalkonium chloride, arsenic and cadmium often travelled together within a population and that these resistance determinants could be tracked as they mobilise into different populations [46]. Furthermore, the sheer volume of sequencing done in this study showcases the high throughput capabilities of whole genome sequencing and the declining associated costs of this technology.

To uncover the resistance response of microorganisms to antimicrobials, greater insight is needed. Specifically, with regards to changes within the bacterial cell that gives rise to the resistance phenotype upon antimicrobial exposure. Transcriptomics in the form of Microarray, Real-Time PCR and RNA-Sequencing can give a “snapshot” into gene expression of a microbial cell under certain conditions. This generates unique expression profiles for different antimicrobials, and compares, for example, the genetic response to antibiotic treatment (penicillin) versus the genetic response to biocide treatment (benzalkonium chloride). Curiao and co-workers [47] used transcriptomics to identify multiple routes *Salmonella enterica* Serovar Typhimurium uses to decrease susceptibility to several biocides and antibiotics. Interestingly, this study also revealed various mutations and adaptations to a range of antimicrobials, even without exposure [47].

As many antibiotics and disinfectants have different modes of action, insight into how microbes respond to these compounds can reveal a great deal. The microbial response can show which systems a specific antimicrobial targets in the cell, and in turn, the microbial defence response. This information is vital to elucidate how resistance develops to different compounds and if this response is specific or can bring about reduced susceptibility to a range of antimicrobials. As a result, a unique microbial response profile can be generated for individual antimicrobials. Merchel Piovesan Pereira and co-workers [10] used this approach to evaluate differential expression brought about by exposing *Escherichia coli* to different biocides. After exposure to ethanol the most affected biological processes were an up-regulation in biofilm associated genes and a down-regulation in aerobic growth associated genes [10]. Whereas after chlorhexidine exposure, in the same bacterium, the most affected systems were an up-regulated

biotin metabolism and motility and flagellum groups which were down-regulated [10]. This study created unique differential expression profiles for each biocide tested and went further by comparing short-term to long-term responses for each biocide. Studies like this provide insight into the mode of action of each antimicrobial as well as clues as to how resistance develops. However, a limitation is the inability to differentiate between chromosomally-encoded and plasmid-based genes [48].

Proteomics takes the study of gene expression one step further by creating an overall protein profile of the cell at a particular moment in time. These profiles include information on post-

translational modifications, or non-gene encoding regulatory responses that can affect the microbial adaption, but which are not captured by transcriptomics [49,50]. This technique can also provide valuable insight into any changes in relative protein concentration and which proteins appear in combination under changing conditions, providing both qualitative and quantitative data. Additionally, this technique can be used to study extracellular proteins that are secreted into the intercellular environment [48]. This is done by combining either 2DE difference gel electrophoresis (DIGE) or liquid chromatography-mass spectrometry (LC-MS) with qPCR to reveal any changes in regulation of protein expression as reviewed by Chernov and co-workers [48]. Lai and co-workers [49] used this technology in the study of *Acinetobacter baumannii*, where it was found that phosphorylation of the Ser-90 phosphosite in the catalytic motif of AmpC β -lactamase conferred imipenem sensitivity, whereas the unphosphorylated enzyme conferred imipenem resistance, therefore revealing a previously unde-scribed antibiotic resistance mechanism [49].

The latest piece in the omics puzzle is metabolomics, which utilizes methods such as nuclear magnetic resonance (NMR), MS, and others to provide a picture of the metabolites present within a cell [51,52]. This knowledge can elucidate how the microbial metabolism changes in response to certain antimicrobials over time [51]. As the mode of action for disinfectants is complex and multiple cellular targets are affected, an overall picture of the entire metabolism of the cell can provide the best picture of the complex response required after exposure to an antimicrobial agent. Metabolomics has been used to study the response of *E. coli* to several antibiotics (kanamycin, tetracycline, ampicillin *etc.*) to create unique metabolite profiles for each antibiotic upon exposure [51]. This study revealed how these antibiotics uniquely impact cellular metabolism, revealing information about the mode of action for each antimicrobial and potential routes for resistance development [51].

Currently, little to no information exists on the proteomic or metabolomic study of disinfectants and biocides and this is a promising avenue for future research. Sequencing technology and bioinformatics software are also evolving at a rapid pace to accommodate the integration of different omics datasets and mitigates the limitations of individual techniques. A combination of transcriptomics and proteomics was used to investigate antibiotic resistance and psychrotolerance of *Pseudomonas psychrophile*, this integration revealed an up-regulation of RNA translation, energy metabolism, ABC transporters and the over-expression of cold shock proteins allows for metabolic adaption in stress conditions [53]. However, the applications of these approaches are limited by the databases used to draw their gene/protein annotations and analysis [48]. The use of this technology is further limited by the high cost of sequencing, accessibility to analysis software and the skills required to run the highly complex bioinformatics programs.

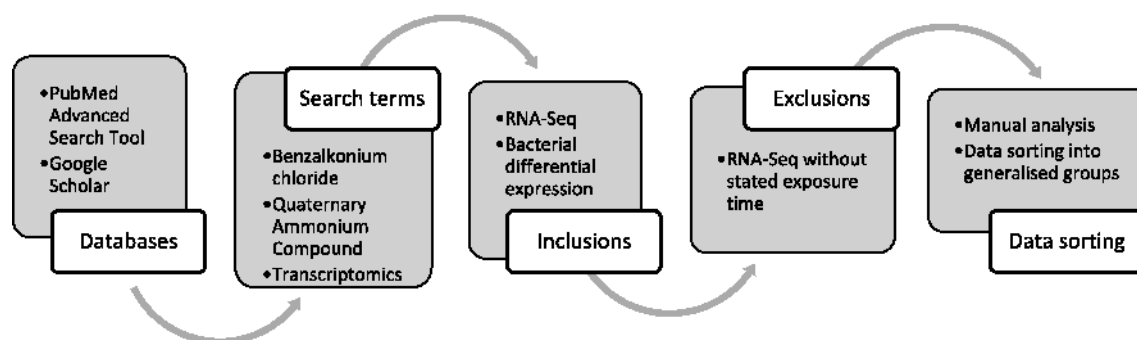


Fig. 1. Literature search and analysis methodology including search terms, databases used and inclusions for search parameters.

5. Transcriptomics: a new technology to tackle an old enemy

5.1. Transcriptomic analysis of the bacterial response to QAC biocide benzalkonium chloride

Transcriptomics is gaining popularity and a great deal of work has been done on the transcriptome of specific bacterial species in response to antibiotics [54-56]. However, significantly less work has been done on biocides and disinfectants. The vast majority of transcriptome work done on biocides focuses on benzalkonium chloride; with individual studies done on DDAC, benzethonium chloride, cetylpyridinium chloride, triclosan, peroxyacetic acid and chlorhexidine [57-60]. Not enough information exists on the transcriptome of any other biocide and as such, this review will focus on BAC only and is not limited to one bacterial species (see Fig. 1).

Transcriptomic analyses have focused on differential gene expression after exposure to benzalkonium chloride in bacteria with a range of susceptibility, this includes *E. coli*, *L. monocytogenes*, *Salmonella* species, *Klebsiella pneumoniae* and *A. baumannii* [10,47,61-63]. A similar methodology is applied where bacteria are exposed to below minimal inhibitory concentration (MIC) levels of benzalkonium chloride and differential gene expression is quantified at different times. Knauf and co-workers [64] published one of the first papers on the response of *A. baumannii* to benzalkonium chloride using Tn-Seq and RNA-Seq techniques. This approach allowed for the mapping of the complete intracellular response to this biocide, from this more insight into the mode of action of BAC could be revealed. Knauf and co-workers [58] hypothesised that benzalkonium chloride does not only target the cell wall of microorganisms but has an equally devastating impact on the proteome of the cell. This is evident in the extent of differential expression in genes functioning in proteostasis (protein folding, aggregated protein turn over and chaperone genes) and suggests there is more to the mode of action of BAC than our current understanding [64].

The general trend amongst all the reviewed literature is a differential expression in four main areas, these are motility, alterations in membrane composition and transport, metabolism adaptations and lastly the stress response depicted in Tables 2-4 [10,11,61,62,64-68]. This review proposes that these four systems are the most affected in the transcriptome and are the most common bacterial response after exposure to BAC.

5.1.1. Differential expression profile of BAC after 30 min (long-term)

Motility is the first of the four main systems differentially expressed after long-term exposure to BAC (>30 min), where a

down-regulation in motility and chemotaxis associated genes is observed [10,11,65]. This includes down regulation of flagella clusters (*fla*, *flg*, *flh* clusters), chemotaxis proteins from the *che* family and motility proteins (*motA*, *motB*) [10,11]. The down-regulation of motility genes after exposure to antimicrobials has

Table 1: Summary of the bacteria and conditions of exposure to BAC reviewed in this paper.

Bacteria	BAC Concentration	Exposure time	Reference
<i>Escherichia coli</i>	3.63 mg/L	30 min & 12 h	[10]
<i>Escherichia coli</i>	0.1 mg/Liter	20 passages of 48 h	[11]
<i>Listeria monocytogenes</i>	1-2 mg/ml	60 min	[58]
<i>Listeria monocytogenes</i>	9.98 mg/L	5 min	[61]
<i>Acinetobacter baumannii</i>	1000 mg/L	30 days	[62]
<i>Acinetobacter baumannii</i>	5 mg/ml	45 min	[64]
<i>Escherichia coli</i>	5120 mg/L	20 h	[65]
<i>Listeria monocytogenes</i>	4.0 mg/ml	120 min	[67]
<i>Escherichia coli</i>	4 mg/L	120 min	[76]

been suggested to play a role in biofilm formation, as reviewed by Guttenplan and Kearns [69]. However, an important conclusion from the review was that additional work is required in both motility and biofilm formation to determine whether a correlation actually exists or not [69]. In contrast, *E. coli* passaged 10 times in BAC, showed a coupled down-regulation in both motility and biofilm formation [11]. For many decades biofilm formation has been seen as an important resistance mechanism for many bacteria [70-73]. However, a review of transcriptomic data seems to contradict this idea and brings into question this resistance mechanism.

In terms of membrane composition and transport, most differential expression includes up-regulation of genes associated with cell envelope maintenance and efflux [11,64-66]. This coupled with a down-regulation of porin genes should result in a reduced intracellular concentration of BAC and limit any intracellular damage brought about by the disinfectant [27,62]. However, two studies of *A. baumannii* after exposure to QAC disinfectants revealed contrasting differential expression results of the porin gene *carO*, one study revealed a down-regulation while the second recorded up-regulation of the porin gene in the same microorganism. Interestingly, an up regulation of sulphate assimilation associated genes might be related to the production of cysteine, an amino acid associated with biofilm adaption and oxidative stress [74,75].

Table 2

Transcriptome profile after bacterial exposure to benzalkonium chloride from literature: Biological systems affected, and genes differentially expressed after 30 min of exposure to BAC. Relevant articles are referenced below the table.

Gene(s)	Description	Expression	Research paper
Motility			
<i>fli, flg, flh</i> clusters	Flagellar	Down	[10,11]
	Flagellar	Down	[11]
	Methyl-accepting chemotaxis protein	Down	[10,11]
<i>ycgR</i>	Chemotaxis	Down	[10,11]
<i>tsr, tar</i>	Motility	Down	[10,11]
	Fimbriae	Down	[65]
	Uncharacterised fimbrial genes	Up	[65]
<i>cheR, cheA, cheB, cheW, cheY, cheZ</i>			
<i>motA,B</i>	Cell envelope porins	Down	[62]
	Cell envelope porin	Up	[64]
<i>papA, papH, ycbR</i>	Electron transport: Cytochrome o oxidase, Formate dehydrogenase, NADH-quinone oxidoreductase	Down	[11]
<i>ydeR*, ydeS*</i>	Carbohydrate transport	Down	[10]
	Molybdate ion transport	Down	[10]
	Putative magnesium transporter	Up	[11]
Membrane composition and transport	Methionine import ATP-binding protein	Down	[10,11]
	L-Ara4N addition to lipid A (polymyxin resistance)	Up	[65]
	Lipopolysaccharide maintenance	Up	[64]
<i>ompF, ompT, ompR, ompW, ompA, carO</i>	Phospholipid maintenance	Up	[64]
<i>carO</i>	Peptidoglycan maintenance	Up	[64]
<i>cyoABACDE, fdoHIG,</i>	γ-cysteine uptake	Up	[65]
	Dipeptide transport	Up	[65]
	Putrescine export	Up	[65]
<i>nuoABACDEFGHIJKLMN</i>	Sulphur utilisation and transport	Up	[65]
<i>IgoT</i>	MFS carbohydrate transport, phosphate transport	Down	[10]
<i>modA</i>	Efflux pump associated genes	Up	[11,64,67,76]
<i>yhiD</i>	Multidrug resistance proteins	Up	[11]
<i>tap</i>	DNA replication	Down	[10]
	Ribosomal RNA	Down	[10]
<i>arnA, arnD, arnT</i>	Regulator: Protein targeting	Down	[10]
	Proteostasis: Protein folding & turnover	Up	[64]
<i>waaH, waaB, wcaJ, galE, pgaC, rfaG, yedE, lpxL, wecB,</i>	Ribosomal maturation	Up	[10]
	Chaperones	Up	[64]
	Translation	Up	[64]
<i>mfaC, mfaE, mfaF</i>	Iron homeostasis	Up	[64]
<i>murL, pbpG, ponA</i>			
<i>ydjN</i>	Regulator stress response	Down	[67]
	Capsule formation	Up	[64]
	Cytolysis	Down	[10]
<i>dppC</i>	ROS defence	Up	[64]
<i>sapC, sapD</i>	Uncharacterised proteins	Up	[11]

Multidrug efflux pumps are well-known mechanisms of antimicrobial resistance and have been found to play a significant role in resistance to disinfectants as reviewed by Chitsaz and Brown [25]. Sub-MIC exposure to BAC is associated with an increase in expression of genes encoding and regulating efflux pumps [11,64,76]. Work done by Forbes and co-workers [11], Fernández-Cuenca and co-workers [62], Knauf and co-workers [64] and Short and co-workers [77] revealed the same trend, a decreased susceptibility to BAC due to decreased expression of outer membrane proteins (*carO*, *ompA*) and an increase in efflux systems (*adeA*, *abeS*, *abeB*, *aceI*, *adeABAC*, *amvA*).

The efflux families most often up-regulated upon QAC exposure are the SMR, PACE, RND and MFS superfamilies, sometimes up-regulated up to 17-fold, with little to no change detected in ABAC families [24,77]. This resistance can be reversed by the use of specific efflux inhibitors such as PabN which targets RND efflux pumps and was able to reverse QAC resistance in *E. coli* [76]. This is one trend the vast majority of

papers have in common and can arguably be described as the main mechanism of decreased susceptibility to biocides. However, when comparing the bacterial response to different biocides, unique differential expression profiles for each biocide are being revealed. Merchel Piovesan Pereira and co-workers [10] compared the differential expression profile of *E. coli* after exposure to ten different biocides. Surprisingly, this study revealed that after exposure to various biocides multidrug efflux pumps were up-regulated in response to BAC, chlorophere and chlorhexidine only and not the other seven biocides. Multidrug efflux pumps were not a general resistance response to all biocides as expected. This is a direct contradiction to the widely accepted notion that efflux systems are the main mechanism of biocide resistance and these contradicting results raise more questions [60].

Cell envelope maintenance is another priority in the bacterial response as multiple genes in this process are up-regulated as seen in Table 1. Additionally, increased expression of *arnA*, *arnD*, *arnT*

Table 3

Transcriptome profile of bacterial exposure to benzalkonium chloride: Biological systems affected, and genes up-regulated within 30 min of exposure to BAC with at least 2 fold change.

Gene(s)	Description	Research paper
Membrane composition and transport		
<i>malE, malF</i>	Phospholipid transport	[10]
<i>znuB, znuC</i>	Zinc ion transport	[10]
<i>pstB, pstC, phoU</i>	Phosphate ion transport	[10]
<i>mgta, chaA</i>	Cation transport	[10]
<i>mepM</i>	Cell wall/peptidoglycan organisation	[10]
<i>acrAB, mdrL, ide</i>	Multidrug efflux pump	[10,61]
<i>lpxP</i>	Lipid A biosynthesis	[10]
Metabolism adaption		
<i>dnaK, dnaJ, groL, hscA, cpxP, clpB</i>	Chaperones and co-chaperonins	[10]
<i>secA</i>	Protein transport	[10]
<i>iscU, iscS, hscB</i>	Iron-sulphur cluster: protein maturation	[10]
<i>iscR, hha, rstA</i>	Regulation of transcription	[10]
<i>ykgO, raiA</i>	Regulation of translation	[10]
<i>rstB</i>	Protein phosphorylation	[10]
<i>opgB</i>	Glucan biosynthesis	[10]
<i>argG</i>	Amino acid/arginine metabolism	[10]
Stress response		
<i>hslU, htpX, groS, hslV, degP, htpG</i>	Response to damage to proteome (misfolding, proteolysis)	[10]
<i>ybjX, pstS</i>	Response to DNA damage	[10]
<i>acrR</i>	Response to drug	[10]
<i>sigB</i>	Response to stress	[58]
Uncharacterised proteins		
<i>yrbL, yebO, yebE</i>		[10]
<i>ybjG</i>	Putative undecaprenyl-diphosphate: bacitracin resistance	[10]

can lead to polymyxin resistance and reduced susceptibility to positively charged antimicrobials, such as QAC disinfectants, by increasing the positive charge of the cell membrane [65,78]. Several genes involved in lipopolysaccharide (LPS) (*waaH, wcaJ, galE*), phospholipid (*mfaC, mfaE, mfaF*) and peptidoglycan (*murL, pbpG, ponA*) maintenance are up-regulated [64]. In addition, genes associated with electron transport were found to be down-regulated including *cyoABACDE*, *fdoHIG* and *nuoABACDEFGHIJKLMN*, indicating a shift in bacterial metabolism away from non-essential functions and to priority responses [11].

Adaptions to the bacterial metabolism after exposure to BAC is another overarching theme in differential expression profiles. The majority of genes differentially expressed are involved in proteostasis, which is the maintenance of a functional cellular proteome [10,64]. This includes numerous genes encoding for protein chaperones (*dsbA, secB, clpB*), ribosomal maturation (*rbfA, rsgA, rluD*), translation and general proteostasis (*clpP, pepP, htpX*) [10,64]. In addition, several genes involved in iron homeostasis were up-regulated such as *feoA, iscR* and *iscA* [64].

Finally, a stress response was the last general trend in the longterm differential expression profile of bacteria exposed to BAC [10,64,67]. This response includes numerous genes playing a role in ROS defence (*oxyR, msrA, grxC*) [64], capsule formation (*cpsG*) [64], stress response regulators (*sigB*) [67] and cytolysis (*yqfA*) [10]. Uncharacterised proteins are common in transcriptomics results as there is substantial work needed in terms of gene and protein annotation, especially in the study of resistance determinants for biocide resistance. Several genes from the *yhi* family were up-regulated in response to BAC, and these genes provide an avenue for future research and further characterisation [11]. In terms of

virulence genes, no general pattern has been detected. Differential expression has been seen in fimbrial genes (mostly down-regulated together with motility associated genes), up-regulation (*iap, sprD*) and down-regulation (*prfA, hly*) of general virulence genes [67].

Table 4

Transcriptome profile of bacterial exposure to benzalkonium chloride: Biological systems affected, and genes down-regulated within 30 min of exposure to BAC with at least 2 fold change compiled by work done by Merchel Piovesan Pereira, Wang and Tagkopoulos on *E. coli* [10].

Gene(s)	Description
Motility	
<i>fgABACDEFG</i>	Flagellum-dependent motility
<i>fiZ, fiG, fiL</i>	Flagellum dependent motility
<i>fhC</i>	Flagellum regulator
Membrane composition and transport	
<i>gatABAC, malE, manXYZ, rbsB, ptsG</i>	Carbohydrate transport
<i>glpT, glpC</i>	Glycerol transport
<i>uraA, codB</i>	Pyrimidine transport
<i>ompF</i>	Drug transport
<i>nmpC, artJ, glpF, sbp, focA</i>	Transmembrane transport
Metabolism adaptions	
<i>carAB, pyrB, codA, pyrI, upp, pyrD</i>	Pyrimidine biosynthesis
<i>sucA, sucB, sucC, sucD, sdhA, sdhB</i>	Tricarboxylic acid cycle
<i>cysI, cysC, cysN, cysH</i>	Sulphate assimilation
<i>glpA, glpB, glpQ, glpK</i>	Glycerol metabolism
<i>gatD, gatZ, gatY</i>	Galactitol metabolism
<i>argI</i>	Ornithine carbamoyltransferase subunit I

<i>aldA</i>	Lactaldehyde dehydrogenase
Stress response	
<i>grcA</i>	Threonine catabolism, response to stress
<i>yagU</i>	Response to acidic pH
<i>ridA</i>	Amino acid biosynthesis, response to toxic substance
<i>efeB</i>	Detoxification, response to DNA damage
<i>efeO</i>	Response to toxic substance, oxidative stress

6

This suggests that increased virulence is not coupled to decreased susceptibility to benzalkonium chloride in resistant isolates.

After reviewing which changes were present in gene expression after exposure to BAC, what is equally interesting is the changes that were not present. For example, biofilm formation has long been regarded as a primary mechanism of antimicrobial resistance as reviewed by Mah and O'Toole [24] and Leung and co-workers [80,80]. However, differential expression of biofilm-associated genes is not mentioned in studies of the bacterial response to BAC. Although, biofilm formation and the genes responsible are poorly understood, this contradiction should be prioritised in future research. In addition, there is little to no mention of the genes implicated in the degradation or metabolism of QAC antimicrobials, the most recently described mechanism of QAC resistance [81e83]. Furthermore, little literature exists on differential expression of genes involved in horizontal gene transfer. As, arguably, the main method of evolution of a resistant population as reviewed by Mc Carlie and co-workers [39], one would expect for genes associated with horizontal gene transfer to be differentially expressed.

To summarise the general trends of long-term (>30min) bacterial response to BAC, systems up-regulated include efflux-associated genes, peptidoglycan and LPS maintenance, proteo-stasis, sulphate assimilation pathway, ROS defence and iron homeostasis. The systems down-regulated after exposure include motility associated genes, cell envelope porins and electron transport associated genes.

5.2. Differential expression profile in response to BAC up to 30min after exposure (short-term)

A common response in differential expression after short term (<30 min) exposure to BAC is up-regulation of ion transport, this includes zinc ions (*znuB*, *znuC*), phosphate ions (*pstB*, *pstC*, *phoU*), and general cation transport (*mgtA*, *chaA*) [10]. As with the long term response, maintaining the cell envelope integrity is a priority as phospholipid transport (*malE*, *malF*) and cell wall/peptidoglycan organisation (*MepM*) are up-regulated as well [10]. As expected, multiple efflux pump associated genes are up-regulated as an initial response to BAC exposure [10,84]. Additionally, the biosynthesis of lipid A endotoxin is up-regulated (*lpxP*) forming part of the bacterial defence response.

In terms of metabolic adaptations, the metabolism shifts to maintaining the integrity of the proteome. This is evident in the up-regulation of chaperone and cochaperones (*dnaK*, *dnaJ*, *groL*, *hscA*, *cpxP*, *clpB*), protein transport (*secA*), Iron-sulphur cluster (protein maturation) (*iscU*, *iscS*, *hscB*), protein phosphorylation (*rstB*), as well as transcription (*iscR*, *hha*, *rstA*) and translation regulation (*ykgO*, *raiA*) [10].

According to current literature, the stress response within the first 30 min of exposure to BAC shows similar patterns to metabolic adaptations as the majority of genes up-regulated function in response to proteome damage (*hslU*, *hpxX*, *groS*, *hslV*, *degP*, *hpxG*) [10]. This response is

elicited together with a response to DNA damage (*ybjX*, *pstS*), drug response (*acrR*) and the general stress response (*sigB*) [10,61]. This oxidative ROS response is implicated as the trigger for other cellular systems to bring about the bacterial response leading to antimicrobial resistance [85].

The systems found to be down-regulated systems in the short term response to BAC are genes associated with flagellum motility (*fgABACDEFG*, *fiZ*, *fiG*, *fiL*, *fhC*), carbohydrate transport (*gatABAC*, *malE*, *manXYZ*, *rbsB*, *ptsG*), glycerol transport (*glpT*, *glpC*), pyrimidine transport (*uraA*, *codB*), drug transport (*ompF*) and transmembrane transport (*nmpC*, *artJ*, *glpF*, *sbp*, *focA*) [10]. This down-regulation of molecule transport presumably coincides with the shift of metabolism away from non-essential functions.

As part of the initial response to BAC, the transport systems of some substrates are down-regulated together with the metabolic pathways that produce of these compounds. These include galactitol metabolism (*gatD*, *gatZ*, *gatY*), pyrimidine biosynthesis (*carAB*, *pyrB*, *codA*, *pyrI*, *upp*, *pyrD*) and glycerol metabolism (*glpA*, *glpB*, *glpQ*, *glpK*) as cellular systems shift in response to BAC [10]. Several genes associated with the tricarboxylic acid cycle (TCA) (*sucA*, *sucB*, *sucC*, *sucD*, *sdhA*, *sdhB*) were also found to be down-regulated suggesting this cycle has no role to play in the cellular defensive response [10]. This aligns with observations in *Acinetobacter olei-vorans* where a metabolic shift from the TCA cycle to the glyoxylate shunt pathway, together with biofilm formation, was found in response to antibiotic exposure [88]. Finally, differential gene expression is observed within the bacterial stress response system comprising of genes involved in threonine catabolism (*grcA*), response to acidic pH (*yagU*), amino acid biosynthesis (*ridA*), detoxification (*efeB*) and response to toxic substance (*efeO*) [10]. As these genes are down-regulated, they may play a role in regulating stress response, however, the bacterial stress response system is currently poorly characterised.

To summarise the reported short-term response to BAC based on differential expression, the systems that are up-regulated and therefore assumed to play a role in the bacterial response include an oxidative ROS response, efflux pumps and proteostasis (Table 3) while the systems responsible for flagellum-associated motility, membrane transport and the TCA cycle are down regulated (Table 4).

5.3. Temporally conserved changes to bacterial expression profile in response to BAC

Maintained responses throughout short and long-term exposure include an up-regulation of efflux associated genes, protection of the proteome, alterations to cell membrane charge, stress defence response and changes to the sulphur-iron metabolism; this is coupled with a down-regulation of motility, as summarised in Tables 2e4 This review proposes these changes can be characterised as general bacterial responses against BAC.

Interestingly, there was no change in biofilm associated genes but there was a down-regulation in motility genes which can be indicative of biofilm formation as reviewed by Guttenplan and Kearns [56]. A biofilm's ability to reduce susceptibility has been well documented as reviewed by Mah and O'Toole [29] and for BAC a positive correlation between biofilm formation capacity and BAC resistance was found in *E. coli* by Sun and co-workers [89]. However, a lack of differential expression of biofilm-associated genes after exposure to BAC suggests that biofilms do not form in response to this antimicrobial. Furthermore, a study

by Forbes and co-workers [11] adapted *E. coli* to BAC, and saw reduced biofilm formation when cultivated in sub-lethal levels of BAC. One explanation could be that a biofilm needs to be established before treatment with an antimicrobial to reduce susceptibility and protect the microbial cells within. Therefore, if a microbial population exists in an established biofilm, it will have some level of protection, but if a microbial population is not in a biofilm already, a biofilm will not be created after exposure to BAC. Thus, biofilm formation is not a response to BAC treatment and will not be differentially expressed after exposure.

Oxidative and acid stress responses have been proposed to play a role in resistance to biocides such as RpoS-dependent genes [90]. The transcriptomic data presented here suggests that the stress response has a much bigger role to play than anticipated. ROS-defence and iron homeostasis is prioritised as part of the stress response together with acid stress after long-term exposure [10,64]. As a maintained common response throughout BAC exposure, it seems this response may be as important as any in reducing susceptibility to BAC. However, the mechanisms of how the stress response reduces susceptibility to BAC remains poorly characterised.

This bacterial transcriptomic response profile generated for BAC is unique as the bacterial response by differential expression differs between biocides [10]. This suggests that mechanisms of biocide resistance may be unique to each biocide and not applicable as a general defence. This was seen in *E. coli* after exposure to ten different biocides, where different differential expression responses were recorded among the biocides used [10]. One example was that of multidrug efflux pumps, where after exposure, an up-regulation in the expression of these systems was seen in only chlorophene, benzalkonium chloride, and chlorhexidine; whereas the remaining seven biocides (ethanol, hydrogen peroxide, sodium hypochlorite, povidone-iodine, isopropanol, glutaraldehyde and peracetic acid), did not invoke differential expression of these systems [10]. This indicates that multidrug efflux pumps are not the general biocide resistance response as currently understood. Furthermore, differential expression of bio-film associated genes was seen after exposure to all biocides (ethanol, chlorhexidine, chlorophene, sodium hypochlorite, povidone-iodine, isopropanol, glutaraldehyde and peracetic acid) except BAC and hydrogen peroxide [10]. This work by Merchel Piovesan Pereira and co-workers [10], together with the transcriptomic profile generated here, adds to the idea that reduced susceptibility is not brought about by general resistance mechanisms but may be a tailor-made response for each specific antimicrobial.

6. Conclusions

When looking at antimicrobial resistance, the mode of action of the biocide needs to be considered as resistance is inferred by countering the microbicidal effects. After a review of the differential gene expression profile, it is evident that several contradictions exist between the current understanding of the mode of action and resistance mechanisms against BAC. When considering the mode of action of BAC, current literature states the target of this compound is the cell wall of microorganisms, but the metabolism shift to proteostasis and widespread stress response suggests the presence of additional intracellular targets.

In addition, analysis of genes involved in well-established biocide resistance mechanisms raised a few questions. The primary biocide resistance mechanism currently is efflux systems, and although multidrug efflux pumps were up-regulated by BAC, work on other biocides suggests this may be a specific response and not a general response as understood. Therefore, efflux systems should be characterised as a targeted response against BAC and not a general biocide resistance mechanism. Furthermore, it seems the stress

response has been greatly underestimated on its impact in biocide resistance. The stress response is observed throughout transcriptomic signatures in response to BAC but this system is poorly characterised and further work is needed to understand how reduced susceptibility to BAC is brought about. Additionally, the lack of differential expression of biofilm associated genes is surprising and suggests biofilm formation may not be a resistance mechanism as currently understood.

This review has generated a transcriptomic profile for bacterial response to BAC. Analysis of the differential gene expression in response to BAC can be related back to how this biocide works and then further inferred to reveal mechanisms employed by bacteria to reduce susceptibility to BAC, and evolve resistance. This review is restricted by the limits of transcriptomic technology and future work should include a range of techniques.

If the mechanisms of resistance are understood, current biocides can be safeguarded, and this knowledge can be used in the development of novel biocide formulas. The development of resistance cannot be stopped altogether, but if we see the antibiotic resistance crisis as a foreshadowing to biocide resistance it is clear that more work needs to be done to remain ahead of this world-wide problem.

Funding

This work was supported by the University of the Free State.

Declaration of competing interest

The authors have no conflict of interest to disclose.

Acknowledgements

The authors would like to acknowledge the University of the Free State for support.

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1.3 Chapter 1 Conclusions

In this chapter, a transcriptomic profile unique to BC has been created for the first time. Research has shown that DGE profiles are different between disinfectants (Merchel Piovesan Pereira, Wang and Tagkopoulos 2020), thus the bacterial response is unique depending on the antimicrobial. However, the transcriptomic signature created does not align well with the current mechanisms of resistance to QAC disinfectants. Current mechanisms of QAC resistance include alteration to cell envelope composition, efflux systems, biofilm formation, modification of QAC compounds and degradation nor metabolism of QACs. Whereas, differential gene expression is found in systems related to alteration to cell envelope composition, efflux systems, proteostasis and the oxidative stress response. This raises the question of whether our current understanding of bacterial resistance mechanisms is correct. Although, it is important to note that this view is limited to a transcriptomic molecular level only, and may not strictly translate to the resistance phenotype. Therefore, further analysis is required to determine what does contribute to the resistance phenotype.

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Chapter 2: Analysis of the growth kinetics of *Serratia* sp. HRI and *Serratia marcescens* ATCC 13880 at sub-MIC levels of QAC-based disinfectants

2.1. Introduction

To elucidate more on the resistance mechanisms of *Serratia* sp. HRI, differential gene expression, in the form of RNA-Seq was planned. To perform RNA-Seq, RNA extraction needs to be done in the exponential phase of growth, and after the contact time with the QAC disinfectant. Thus, growth analysis was conducted on *Serratia* sp. HRI to reveal the optimum time for RNA extraction and downstream analysis. However, during the growth analysis, when the QAC disinfectants were added to the growth medium, an unexpected increase in maximum specific growth rate was observed for *Serratia* sp. HRI; later identified as the hormetic effect. The hormetic effect is a stimulation of growth rate at low concentrations of antimicrobials due to an overreaction of the stress response (Morales-Fernández *et al.* 2014; Tauffenberger *et al.* 2019). This has been observed once before for disinfectants in *Staphylococcus aureus* (Morales-Fernández *et al.* 2014). This experimental chapter has been published and is entitled “The Hormetic Effect Observed for Benzalkonium Chloride and Didecyldimethylammonium Chloride in *Serratia* sp. HRI” in the journal *Microorganisms*. This is a peer-reviewed, accredited scientific journal. As the first author, I researched the topic, designed the methodology, conducted all experiments, wrote, and compiled the article. The co-authors on this article are my two promoters, namely, Prof. R. R. Bragg and Dr. C. E. Boucher-van Jaarsveld, together with three academics Laurinda Steyn, Dr. Louis L. du Preez and Dr. Julio Castillo Hernandez who provided guidance on methodology design and data analysis. This published original research paper is presented as the complete second chapter of this dissertation.



The Hormetic Effect Observed for Benzalkonium Chloride and Didecyltrimethylammonium Chloride in *Serratia* sp. HRI

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Abstract: Hormesis, or the hormetic effect, is a dose- or concentration-dependent response characterised by growth stimulation at low concentrations and inhibition at high concentrations. The impact of sub-lethal levels of disinfectants on the growth of *Serratia* species is critical to understanding the increasing number of outbreaks caused by this pathogen in healthcare settings. *Serratia* sp. HRI and *Serratia marcescens* ATCC 13880 were cultivated in sub-lethal levels of benzalkonium chloride (BAC), Didecyltrimethylammonium chloride (DDAC), and Virukill™. The maximum specific growth rates, doubling times, and cell counts were compared. The results revealed significant increases in maximum specific growth rates and shorter doubling times for *Serratia* sp. HRI when cultivated in sub-lethal levels of BAC and DDAC. The significant stimulatory effect of sub-lethal levels of these disinfectants for *Serratia* sp. HRI represents the first time hormesis has been observed in a Gram-negative bacterium for any disinfectant. Furthermore, this study is the first to observe the hormetic effect after treatment with DDAC and the second study to date analysing the impact of sub-lethal levels of disinfectants on the growth of

Keywords: quaternary ammonium compounds; antimicrobial tolerance; growth analysis



Citation: McCarlie, S.J.; Steyn, L.; du Preez, L.L.; Boucher, C.E.; Hernandez, J.C.; Bragg, R.R. The Hormetic Effect Observed for Benzalkonium Chloride and Didecyltrimethylammonium Chloride in *Serratia* sp. HRI. *Microorganisms* **2023**, *11*, 564. <https://doi.org/10.3390/microorganisms11030564>

Academic Editor: Miguel Viñas

Received: 1 February 2023

Revised: 10 February 2023

Accepted: 21 February 2023

Published: 23 February 2023



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Introduction

Growth analysis of *Serratia* species has focused on the growth kinetics and associated production of the red pigment, prodigiosin, by members of the *Serratia marcescens* species [1]. Prodigiosin production is associated with a faster growth rate and increased biomass due to coupled ATP production in pigment-producing strains of *Serratia* species (such as *Serratia marcescens* ATCC 13880) [2]. In contrast, non-pigmented strains (such as *Serratia* sp. HRI) are associated with a lower growth rate and cell density over time compared to pigmented strains. Little information exists on the effect of disinfectants on the growth of *Serratia* species. As a nosocomial pathogen increasingly associated with outbreaks in healthcare facilities [3–7], the impact of sub-lethal levels of disinfectants on the growth of this pathogen is crucial to direct hygiene and safety standards in the future.

As early as 1981, *S. marcescens* was identified as the cause of nosocomial outbreaks, often isolated from the same bottles of disinfectants or antiseptics responsible for preventing these outbreaks [8]. *S. marcescens* was isolated from a 2% chlorhexidine solution after 27 months of storage [8]. Not only was this isolate able to survive for prolonged periods in the disinfectant solution, but it was able to survive in concentrations up to 20 g/mL, 20 times higher than the minimal inhibitory concentration (MIC) value (1024 g/mL) recorded for this isolate [8].

In terms of Quaternary Ammonium Compound (QAC) exposure at sub-MIC levels, current literature shows either no significant effect or an inhibitory effect on bacterial growth rate and cell density [9,10]. Recent studies found no significant difference in growth rates for *Escherichia coli* and *Listeria monocytogenes* respectively, after exposure to sub-MIC levels of benzalkonium chloride (BAC) when compared to unchallenged controls [9,11].

Whereas for *S. marcescens*, cell density decreased for both resistant and susceptible strains, even at the lowest concentration of BAC [12]. This phenomenon has also recently been observed in *Bacillus cereus* and *Escherichia coli*, where the addition of sub-MIC levels of Didecyltrimethylammonium chloride (DDAC) resulted in a reduction in optical density (OD) and CFU/mL values coupled with a lower growth rate compared to positive controls (no disinfectant added) [10]. In contrast, significant stimulation of growth after the addition of disinfectants at a low concentration has been observed in one instance by *Staphylococcus aureus* [13]. The hormetic effect is the growth stimulation by adaptive responses triggered by low concentrations of antimicrobials [13,14]. This phenomenon has been associated with various antibiotics and metal nanoparticles [14–18], but limited information exists on whether this applies to disinfectants and, if so, which active ingredients. Hormesis was observed for *S. aureus*, where a significant increase in growth rate was recorded for low chlorhexidine digluconate, povidone iodine, and BAC concentrations. A similar trend of increased growth rate was observed for *Pseudomonas aeruginosa* for all three disinfectants at low concentrations, but the changes were insignificant. This work suggests that the hormetic effect does apply to disinfectants, but it has only been confirmed in *S. aureus* and no other bacterial species yet.

The impact of the hormetic effect is far-reaching in any industry that relies upon disinfection and cleaning protocols. In areas with limited accessibility (air vents), high organic load (surgical wounds), or biofilms (infant feeding bottles) [3], hormesis could have devastating effects. The cleaning and disinfection of these areas and products with antiseptics or disinfectants at incorrect concentrations could cause stimulation of microbial growth instead of an inhibitory effect [2]. Therefore, the hormetic effect could have severe and far-reaching consequences for healthcare systems and infection control.

This study compared the maximum specific growth rate and doubling times of *Serratia marcescens* ATCC 13880 type strain (pigment-producing) and the antimicrobial-resistant isolate *Serratia* sp. HRI (non-pigment producing) at sub-lethal levels of three QAC-based compounds benzalkonium chloride (BAC), didecyltrimethylammonium chloride (DDAC), and Virukill™. To date, some work has been done on the effect of either DDAC or BAC on the growth of bacteria [9–11,13]; however, to our knowledge, this study is the first to compare the effects of different QAC-based disinfectants to each other on the same microorganism. In addition, little to no research has been done on the effect of multiple QAC-based disinfectants on *Serratia* species' growth. An important question is whether disinfectants induce the hormetic effect. This study is the first to confirm a significant hormetic effect induced by these disinfectants for a bacterium other than *Staphylococcus aureus*, and thus is the second study to confirm hormesis after exposure to disinfectants. Furthermore, this is the first time a significant hormetic effect has been observed in a Gram-negative bacterium after treatment with disinfectants.

2. Materials and Methods

2.1. Microorganisms

The microorganisms used in this study were *Serratia* sp. HRI, isolated from a bottle of DDAC-based disinfectant [19], and the closest related type strain, *Serratia marcescens* subsp. *marcescens* ATCC 13880, obtained from the American Type Culture Collection.

2.2. Disinfectants

Benzalkonium chloride obtained from Sigma-Aldrich ($\geq 50\%$ in water), Didecyltrimethylammonium chloride (DDAC) obtained from ICA International Chemicals (80% UNIQUT), and Virukill™ (Poly Dimethyl Ammonium Chloride 120 g/L). The MIC levels of these disinfectants for *Serratia* sp. HRI and *Serratia marcescens* subsp. *marcescens* ATCC 13880 were previously reported [19], and sub-MIC levels for this study were between 10% and 50% of the MIC level for each bacteria.

2.3. Inoculum and Shake Flask Preparation

Inocula of *Serratia* sp. HRI and *S. marcescens* ATCC 13880 were prepared by transferring a single colony from 24 h agar plates to 25 mL LB medium (10 g/L Merck Tryptone, 10 g/L Merck sodium chloride, 5 g/L Merck yeast extract) in 250 mL Erlenmeyer flasks and incubated at 37 °C for 16 h. From this pre-inoculum, 2.5 mL was added into 500 mL Erlenmeyer flasks containing LB media supplemented with one of the three QAC-based disinfectants (benzalkonium chloride, Virukill™, and DDAC) and incubated at 250 r min⁻¹ and 37 °C. The growth analysis of *Serratia marcescens* ATCC 13880 was performed at a sub-MIC level of 0.0001% (final volume) for all three disinfectants for three biological replicates. The sub-MIC levels of disinfectant for *Serratia* sp. HRI were 0.01% (final volume) for each disinfectant during growth analysis. After adding the disinfectant to the LB media (before inoculation), a blank reading was taken and subtracted from consequent optical density (OD) measurements. Blank readings for each sample were taken after the disinfectant was added, as the high amount of disinfectant in the media impacted the OD reading. Subsequently, an absorbance spectrum was generated for the three QAC-based disinfectants, as depicted in Figure A1.

Three controls were added, the first comprising each bacterium in growth media without disinfectant; the next control was included with no inoculum or disinfectant to rule out any contamination; and the last control contained disinfectant but no inoculum to ensure any change in absorbance over time was due to the culture and not interactions of the disinfectant. Three biological replicates were done for each experimental condition.

2.4. Analytical Methods

Optical density was measured with a PhotoLab S6 spectrophotometer at 690 nm. The culture was diluted when the OD exceeded 0.8. The maximum specific growth rate and doubling time of a batch culture were determined by linear regression analysis with Microsoft Excel (Microsoft Corporation, WA, USA) of the exponential phase of the growth curve. Multiple paired t-tests were performed (Paired Two Sample for Means) to determine whether there were significant differences in each bacterium's maximum specific growth rates. A *p*-value ($P(T \leq t)$ two-tail) less than 0.05 indicated a statistically significant difference with 95% confidence.

2.5. 60-Min Growth Analysis with Cell Count

Samples were prepared and run as indicated above with sub-MIC levels of disinfectant. At 10-min intervals from time zero, OD (690 nm) readings were taken for each sample until 60 min. In addition to an OD measurement, cell counts were done at each time point. Samples were serially diluted in sterile water, and dilutions at the 10⁻⁶ and 10⁻⁷ levels were plated out on TSA agar and incubated overnight at 37 °C. After that, CFU/mL was calculated for each sample at each time point. An ANOVA single factor test (Microsoft Corporation, Redmond, WA, USA) was run to determine whether there was a significant difference between the CFU/mL values at each time point for each biological replicate.

2.6. Absorbance Spectrum

QAC disinfectants were prepared at 0.01% in LB media, the highest concentration used for growth analysis. Samples were loaded onto a 96-well plate and analysed using a SpectraMax M2 microtitre plate spectrophotometer, where a range of wavelengths from 200 to 1000 nm were scanned for peaks in absorbance due to the presence of the disinfectant. This was done to determine whether changes in OD at 690 nm could be due to interfering absorbance of the disinfectant molecules and not attributable to the inoculum. The data was uploaded to SoftMaxPro (v. 5.2) and shuffled (blank deducted) before being plotted as an absorbance spectrum in Figure A1.

3. Results

3.1. Growth Analysis of *Serratia* sp. in the Presence of Sub-MIC Level Disinfectants

When cultivated in non-supplemented media, *Serratia marcescens* ATCC 13880 type strain had a lower doubling time and a significantly higher maximum specific growth rate compared to the resistant isolate, *Serratia* sp. HRI is depicted in Table 1.

Table 1. Calculated maximum specific growth rate ($\mu_{\max}$ h⁻¹) and growth kinetics analysis of *S. marcescens* ATCC 13880 and *Serratia* sp. HRI without and after adding three disinfectants (Benzalkonium chloride, DDAC, and Virukill™). A1-3: *S. marcescens* ATCC 13880 biological replicates exposed at 0.0001% of disinfectant; H1-3: *Serratia* sp. HRI biological replicates exposed at 0.01% of disinfectant; A/H+: positive control with no disinfectant added.

Sample	$\mu_{\max}$ (h ⁻¹) (Td, h)	Ave. $\mu_{\max}$	Doubling Time	Ave. Td
No Disinfectant				
A1	1.06	1.07 ± 0.04	0.65	0.65
A2	1.04		0.67	
A3	1.11		0.62	
H1	0.93	0.92 ± 0.01	0.74	0.75
H2	0.91		0.76	
H3	0.92		0.76	
Sub-MIC Level Benzalkonium Chloride				
A1	1.06	1.04 ± 0.04	0.52	0.67
A2	1.00		0.73	
A3	1.07		0.76	
H1	1.61	1.75 ± 0.23	0.51	0.48
H2	1.62		0.51	
H3	2.01		0.41	
A+	0.96	N/A	0.72	N/A
H+	0.89	N/A	0.78	N/A
Sub-MIC Level DDAC				
A1	0.90	0.97 ± 0.01	0.77	0.7634
A2	1.08		0.73	
A3	0.93		0.79	
H1	1.44	1.43 ± 0.01	0.58	0.5172
H2	1.43		0.48	
H3	1.41		0.49	
A+	1.02	N/A	0.69	N/A
H+	0.84	N/A	0.82	N/A
Sub-MIC Level Virukill™				
A1	1.09	1.14 ± 0.05	0.64	0.6075
A2	1.16		0.60	
A3	1.18		0.59	
H1	0.93	0.94 ± 0.04	0.74	0.7353
H2	0.91		0.76	
H3	0.99		0.70	
A+	1.12	N/A		N/A
H+	0.94	N/A		N/A

After adding benzalkonium chloride to the medium, the maximum specific growth rate of the type strain decreased slightly, and the correlating doubling time increased, as expected after adding an antimicrobial. However, adding BAC to the medium resulted in a significantly higher maximum specific growth rate for *Serratia* sp. HRI compared to non-supplemented media depicted in Figure 1.

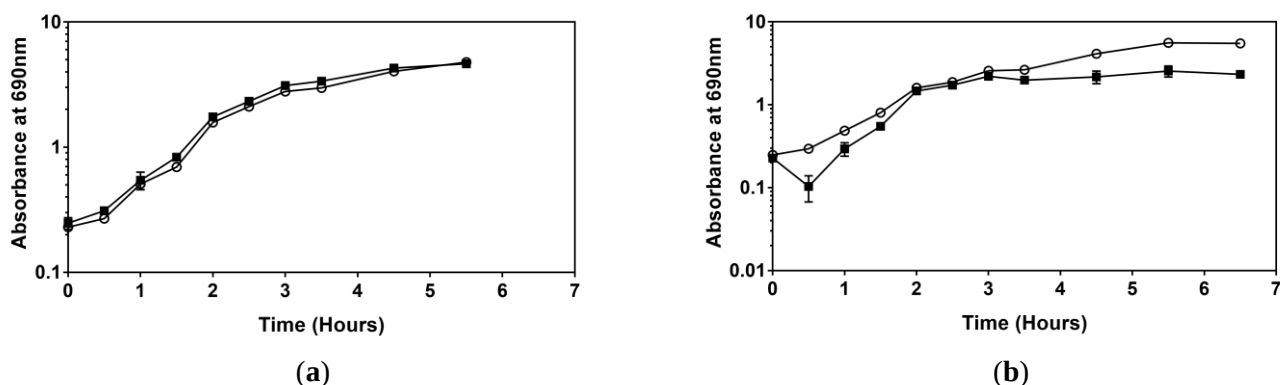


Figure 1. Growth comparison of supplementation with sub-MIC levels of benzalkonium chloride (●) and without BAC (○). For some points, the error bars are shorter than the height of the symbol. In these cases, the error bars were not drawn: (a) *Serratia marcescens* ATCC 13880; (b) *Serratia* sp. HRI.

Similar results were observed after adding DDAC to the medium; for the type strain, the growth rate decreased slightly (Figure 2), but no significant changes were observed. In contrast, sub-MIC levels of DDAC resulted in a significant increase in the maximum specific growth rate for *Serratia* sp. HRI compared to non-supplemented media (Table 1). Furthermore, as with BAC, the growth rate of *Serratia* sp. HRI increased to surpass the type strain after the addition of DDAC. This correlated to a shorter doubling time for the resistant isolate and matched the results after adding BAC to the medium (Table 1).

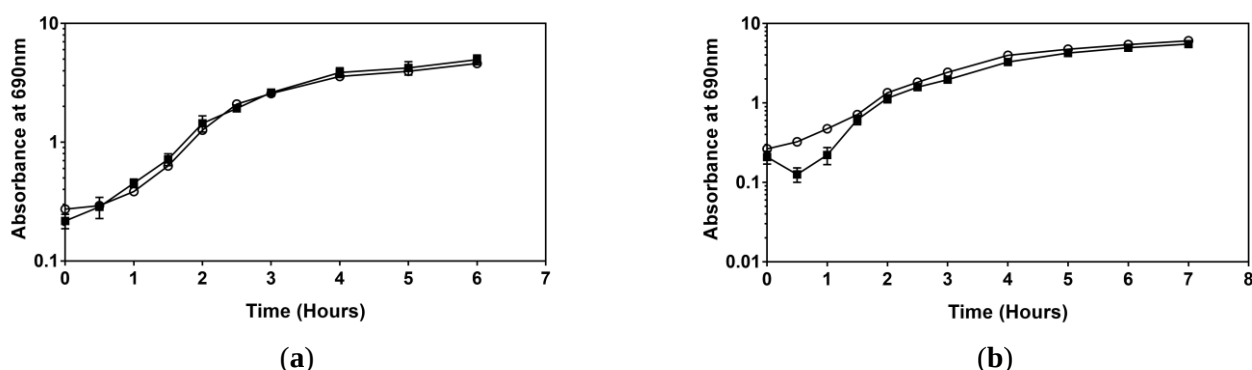


Figure 2. Growth comparison of supplementation with sub-MIC levels of DDAC (●) and without DDAC (○): (a) *Serratia marcescens* ATCC 13880; (b) *Serratia* sp. HRI.

After adding the more recently developed QAC formulation, Virukill™, the results did not follow the trends observed with the older generation disinfectants (BAC and DDAC). After adding Virukill™, as with the control (no disinfectants added to the growth media), the type strain had a significantly higher maximum specific growth rate and a lower doubling time when compared to the resistant isolate depicted in Figure 3. There was also no significant difference between growth in sub-MIC levels of Virukill and the control group of *Serratia* sp. HRI cultured without a disinfectant (Table 1).

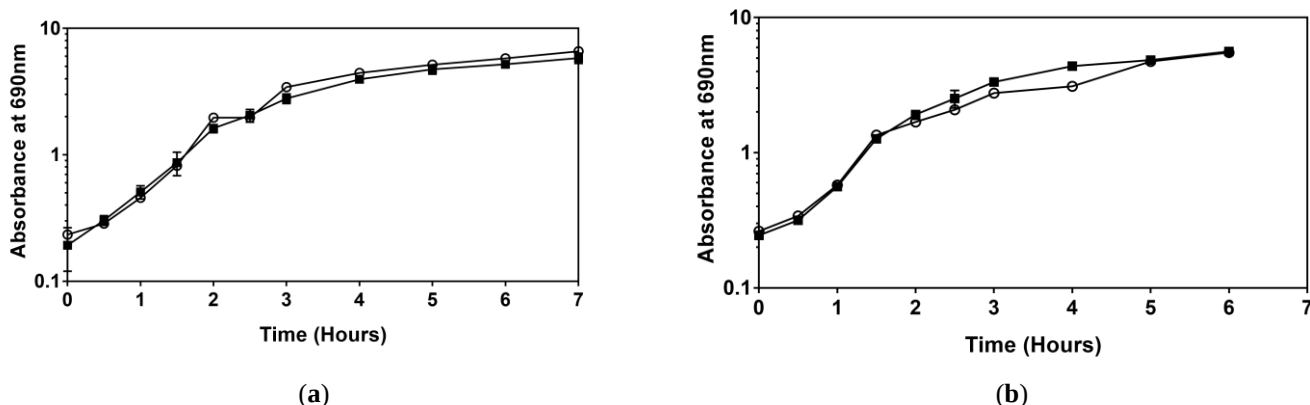


Figure 3. Growth comparison of supplementation with sub-MIC levels of Virukill (●) and without Virukill (○): **(a)** *Serratia marcescens* ATCC 13880; **(b)** *Serratia* sp. HRI.

Interestingly, in the growth curves of *Serratia* sp. HRI after the addition of BAC (Figure 1) and DDAC (Figure 2) a drop in absorbance was observed within the first 30 min of cultivation. This drop in OD was not present for the type strain and did not occur in the positive control of *Serratia* sp. HRI in each experiment. The decrease in absorbance was only observed for the resistant strain when BAC or DDAC was added to the media.

3.2. Growth and Cell Count Analysis of *Serratia* sp. HRI and *Serratia* Marcescens ATCC 13880

It was speculated that the drop in OD at the 30-min mark could be due to cell death after the addition of disinfectants. To test this, growth analyses were repeated, represented by Figure 4, focusing on the first hour of growth and calculating CFU/mL values to determine if the drop in OD could be correlated to a decline in cell count.

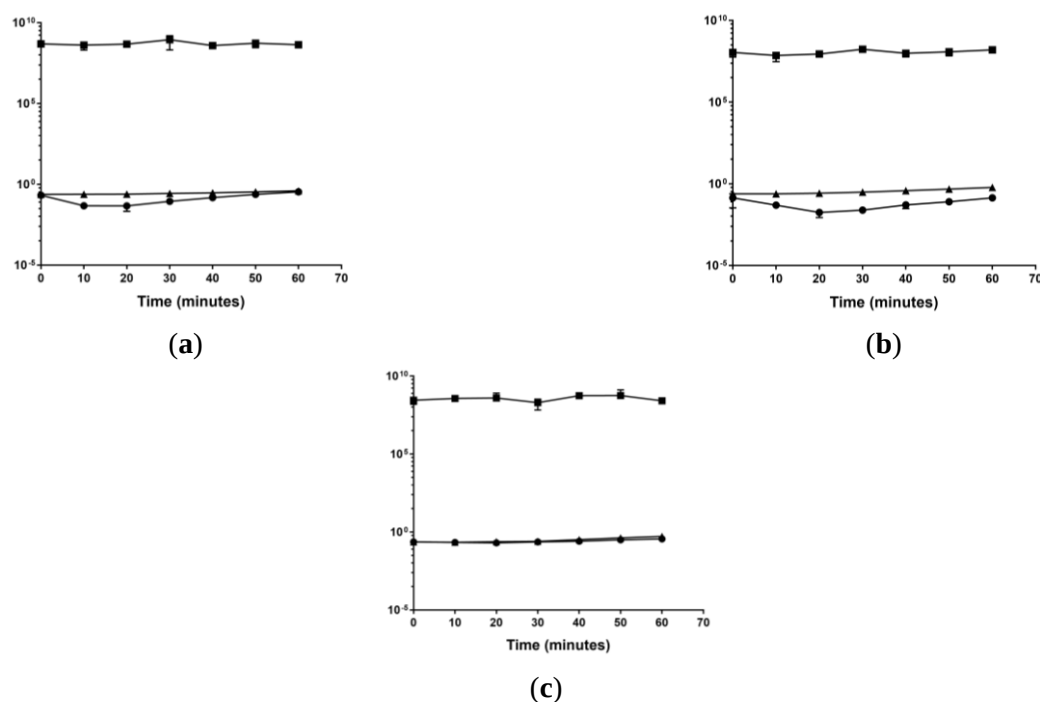


Figure 4. Comparison of changes in absorbance readings (●) and CFU/mL values for *Serratia* sp. HRI (■) as well as CFU/mL values of *Serratia marcescens* ATCC 13800 (▲) over 60 min cultivated in the presence of sub-MIC levels of disinfectants: (a) Benzalkonium chloride; (b) DDAC; (c) Virukill.

Figure 4 indicates that the drop in OD for BC and DDAC seemed to be at its lowest at 20 min before a gradual rise in absorbance started. The CFU/mL values calculated showed no correlation to the drop in absorbance within the first hour of cultivation, which suggests that a drop in cell count is not responsible for the decline in OD readings observed for *Serratia* sp. HRI.

3.3. Absorbance Spectrum of QAC-Based Disinfectants

To account for any changes in absorbance caused by the disinfectant molecules, an absorbance spectrum was generated for the three QAC-based disinfectants. As depicted in Figure A1, absorbance was high, being between 200 nm and 300 nm for all three disinfectants. For growth analysis, readings were taken at 690 nm, where absorbance readings for the disinfectants at 0.01% were as follows: Virukill™ at 0.0464, DDAC at 0.1013, and BC at 0.1433.

4. Discussion

Previous studies on this pathogen have shown that unchallenged pigment-producing strains of *Serratia* species have higher ATP production, cell yield, and specific growth rate compared to strains that do not produce the prodigiosin pigment [2]. This was observed in this study when comparing the maximum specific growth rate and doubling time of *Serratia marcescens* ATCC 13880 (pigment-producing) and *Serratia* sp. HRI (non-pigmented). The lower growth rate of the resistant isolate observed in Table 1 (*Serratia* sp. HRI) is also expected as a lower growth rate can contribute to decreased susceptibility against antimicrobials that target cell membrane synthesis, such as β -lactam antibiotics [20–22]. This also applies to disinfectants, as a lower growth rate aided in reduced susceptibility to benzalkonium chloride in *Pseudomonas aeruginosa* [23]. This indicates that *Serratia*

sp. HRI may have evolved a slower growth rate compared to *Serratia marcescens* ATCC to increase tolerance to some antimicrobials [24].

When analysing growth patterns of the type strain, *Serratia marcescens* ATCC 13880, adding sub-MIC levels of BAC, DDAC, and Virukill™ disinfectants had no significant impact on the growth rate, as illustrated in Figures 1–3. In contrast, after sub-MIC levels of BAC and DDAC were added to the resistant isolate, *Serratia* sp. HRI, the specific growth rate increased significantly in response to both disinfectants compared to unsupplemented media (Table 1). An increase in maximum specific growth rate and cell density after the addition of disinfectants has been recorded once before in *Staphylococcus aureus* exposed to chlorhexidine digluconate, BAC, and povidone iodine [13]. This was the first time the hormetic effect was observed for disinfectant solutions.

The results for Virukill™, illustrated in Table 1, were different from earlier generations of QACs, BAC, and DDAC when added during the cultivation of *Serratia* sp. HRI. Although the active ingredient of Virukill™ is similar to DDAC, unknown additives in this product could be responsible for the extraordinary result. No significant differences were observed after adding Virukill™ during bacterial cultivation, which suggests that new formulations of QAC-based disinfectants do not induce the hormetic effect. This is vitally important in designing and producing new antimicrobial compounds and warrants further study.

Upon closer inspection of the BAC and DDAC growth curves, it was noted that during the cultivation of *Serratia* sp. HRI, there was a drop in absorbance at around the 20-min mark, which evened out again after 50 min (Figure 4). This drop was not present in the growth analysis of *Serratia marcescens* ATCC 13880 and was absent from the *Serratia* sp. HRI positive control; it is only seen when *Serratia* sp. HRI is cultivated in media with sub-MIC levels of BAC (Figure 1) and DDAC (Figure 2). The cell counts depicted in Figure 4 show that the drop in absorbance does not correlate with a drop in CFU/mL values. The negative control containing disinfectant also did not follow this pattern indicating that it is not the disinfectant sticking to the glassware or precipitating out of the media. After adding the disinfectant to the media (0.1%), the absorbance readings increased by about 0.2 units; therefore, if the disinfectant was removed from the media by cell interactions, it would have a significant enough impact to affect the absorbance readings.

The initial drop in absorbance coupled with an increased maximum specific growth rate for *Serratia* sp. HRI has not been observed and reported in the current literature. This brings forward a hypothesis that explains both the drop in absorbance readings within the first 30 min of growth and the increase in the maximum specific growth rate of *Serratia* sp. HRI. The action of QAC-based disinfectants could explain the initial drop in absorbance readings. QAC-based disinfectants bind to the negatively charged cell membranes of bacteria and damage the cell wall [25,26]. This can result in an initial decrease in absorbance readings as the disinfectant is no longer suspended in the media. Thereafter, the increased growth rate could be due to *Serratia* sp. HRI using the disinfectant as an energy source. Bacteria can metabolise QAC-based disinfectants, as observed in *Pseudomonas* species [27,28]. Additionally, several articles have reported the isolation of *Serratia marcescens* from bottles of antiseptics and disinfectants after years of storage without any additional carbon or nitrogen source [8,12,29,30]. The resistant isolate used in this study, *Serratia* sp. HRI, was isolated from a bottle of DDAC-based disinfectant diluted in water, where no other energy sources were present [19]. This study suggests that this isolate can metabolise benzalkonium chloride and DDAC. These antimicrobial compounds may be metabolised into less toxic intermediates and biomass, accounting for the increased growth rate of *Serratia* sp. HRI. The use of QAC-based disinfectant compounds as an energy source elucidates alternative bacterial metabolism pathways, allowing pathogenic bacteria to use antimicrobial compounds as their sole carbon and energy source [31]. Metabolic adaptations include hydroxylation, N-dealkylation, N-demethylation, and β -oxidation of QAC compounds [31–

34]. The metabolism of disinfectants has not yet been observed for *Serratia* species, and future work will be needed to confirm if these compounds are being metabolised or degraded.

The hormetic effect is hypothesised to be due to an overreaction of the bacterial stress response creating a stimulatory effect [35]. This hypothesis is well-founded as these microorganisms are certainly under stress after treatment with antimicrobials. It is interesting to note that this work may provide a more specific mechanism of action of the hormetic effect, which is the metabolism of antimicrobial compounds as an energy source resulting in increased biomass. Whether the metabolism of these compounds is linked to the stress response remains unknown. Nevertheless, this work presents a possible explanation of how the hormetic effect stimulates growth and reiterates the importance of the stress response in bacterial tolerance and resistance. Further work will be required to elucidate whether the metabolism of these compounds plays a role in hormesis and if this is linked to the stress response.

5. Conclusions

The results of this study represent only the second time a significant hormetic effect has been confirmed for disinfectants and the first time a hormetic effect has been observed for disinfectants in a Gram-negative bacterium. This is the first time DDAC has induced the hormetic effect. Interestingly, a hormetic effect was not induced by the more recently developed generation QAC (Virukill™), suggesting that it is possible to design products that inhibit this effect. This implication could direct the design of new antimicrobials and indicates that there may be a solution to this devastating effect. Furthermore, this study provided insight into cell-disinfectant interactions elucidated by changes in absorbance and may provide the first evidence of the metabolism of disinfectants by *Serratia* species.

Serratia marcescens is an opportunistic pathogen increasingly associated with infections and outbreaks in neonatal and paediatric healthcare units [3–7]. Healthcare facilities use disinfectant products daily for infection control, wound care, and to prevent the development of antimicrobial resistance. The observations in this study suggest that incorrectly applied or diluted disinfectant products can have the opposite effect as intended; instead of inhibiting microbial growth, it can be stimulated. In healthcare settings where antimicrobial resistance is a major issue, this could have devastating impacts and lead to increased pathogenic outbreaks. Special care must be taken in areas where microbial growth needs to be controlled, such as operating theatres, wound care, catheters, and surgical instruments. The observations made in this study will be vitally important in preventing future hospital acquired infections and infection control world-wide.

Author Contributions: Conceptualization, S.J.M., C.E.B., and R.R.B.; methodology, S.J.M., L.S.; software, L.S., S.J.M., and J.C.H.; validation, L.S., S.J.M., and J.C.H.; formal analysis, S.J.M.; investigation, S.J.M.; resources, R.R.B. and C.E.B.; data curation, S.J.M.; writing—original draft preparation, S.J.M.; writing—review and editing, S.J.M., L.S., L.D., C.E.B., R.R.B., and J.C.H.; visualization, S.J.M. and L.L.d.P.; supervision, R.R.B. and C.E.B.; project administration, R.R.B.; funding acquisition, R.R.B. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

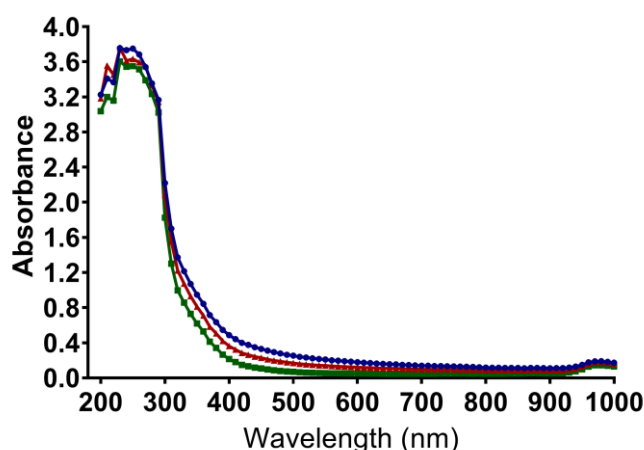


Figure A1. Absorbance spectrum of QAC disinfectants. BC: benzalkonium chloride (●) DDAC: Didecyltrimethylammonium chloride (▲) Virukill™ (■).

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2.3 Chapter 2 Conclusions

The unexpected results from growth analysis revealed the hormetic effect as a troubling consequence for poor cleaning and hygiene control. Incorrect dilutions or improper applications could lead to a biocide being applied at sub-MIC levels which could trigger faster microbial growth (Migliore, Rotini and Thaller 2013; Morales-Fernández *et al.* 2014). This could have deadly consequences in healthcare settings where disinfectants and antiseptics are used regularly, or lead to food-borne illness in food and beverage processing (Merchel Piovesan Pereira and Tagkopoulos 2019). The drop in OD within the first 30 minutes of cultivation of *Serratia* sp. HRI (as seen in Figures 1b and 2b of this chapter), is surprising with no similar results in literature. Due to the high levels of resistance of *Serratia* sp. HRI, the addition of sub-MIC levels of QACs is high enough to increase the optical density when added to the growth medium. The change in OD at 20 minutes (around 0.2), is similar to the observed increase in OD when the disinfectant is added to the growth media. Therefore, this drop in OD could be due to the disinfectant being removed from suspension within the first 20 minutes. This could be the disinfectant interacting with the cell membrane from the outside or from the inside of the cell. Further analysis in Chapter 3 was done to elucidate whether this drop in OD could be due to the disinfectant entering the cells and working from inside.

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Chapter 3: Insights into cellular morphology and biofilm formation in *Serratia* sp. HRI and *Serratia marcescens* ATCC at sub-MIC levels of QAC-based disinfectants

3.1. Introduction

The effect of QAC disinfectants on the growth rate of *Serratia* sp. HRI revealed significant changes between the resistant isolate and the type strain, *Serratia marcescens* ATCC 13880. Interestingly, a notable drop in OD measurements was observed within the first 30 minutes of cultivation in the presence of QACs. This drop in OD was not observed for positive controls where no disinfectant was added, and equated to the change in OD when the disinfectant was added to the growth medium. This suggests that the drop in OD may have been the disinfectant entering the cells and exiting the media. To investigate this further, Nanoscanning Auger Microscopy (NanoSAM) was utilised in an attempt to locate increased nitrogen as a component of the QAC within treated cells in comparison to untreated cells. Additionally, Scanning Electron Microscopy (SEM) was employed to reveal any morphological changes in cells when treated with sub-MIC levels of QACs compared to untreated cells.

This experimental chapter has been submitted for publication and reviewed. The suggested changes from reviewers have been made and this manuscript has been resubmitted to reviewers pending the final outcome. This manuscript of original research is presented as the complete third chapter of this dissertation currently under review at the journal Heliyon. This is a peer-reviewed, accredited scientific journal. As the first author, I researched the topic, designed the methodology, conducted all experiments, wrote, and compiled the article. The co-authors on this article are my two promoters, namely, Prof. R. R. Bragg and Dr. C. E. Boucher-van Jaarsveld, together with two academics E. Coetsee and H. Grobler from the UFS Physics Department (NanoSAM centre) and Centre for Microscopy respectively; who provided guidance on methodology design, data analysis and operated equipment related to the NanoSAM and SEM work.

3.2. Microscopy techniques unveil the effect of QAC-disinfectants on biofilm formation, cell morphology, and localisation of QAC compounds.

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Abbreviations

NanoSAM: nanoScanning Auger Microscopy

SEM: Scanning electron microscope

QAC: Quaternary Ammonium Compound

DDAC: Didecyltrimethylammonium chloride

ABSTRACT

Antimicrobial resistance is a growing world-wide crisis. The recent surge in antimicrobial resistance leading to hospital-acquired infections can be attributed to increased and improper use of biocides during the COVID-19 pandemic. To elucidate more about biocide resistance, NanoSAM and SEM were used to analyse a highly resistant isolate, *Serratia* sp. HRI. These images and atomic percentage analysis revealed changes to cell morphology, biofilm formation and localisation of the disinfectant after treatment with benzalkonium chloride or Virukill® disinfectants. After 20 minutes of treatment, a significantly higher atomic percentage of nitrogen was detected within the treated cells when compared to untreated cells, suggesting the QAC-disinfectants were localised within the cell. This work, together with evidence in literature suggests that QAC biocides do not work from outside the cell and puts forward the hypothesis that instead, QAC biocides cause damage from inside the cell. Additionally, long-term exposure revealed no biofilm formation in the resistant isolate after a 24-hour treatment with the QAC biocides, however, *Serratia* sp. HRI did form many outer membrane vesicles (OMVs). This suggests that biofilm formation is not attributing to the extremely high levels of resistance in this isolate. Evidence gathered in this study, together with further evidence in literature suggest that biofilm formation is not responsible for resistance to QACs but instead OMVs act as a physical barrier to protect cells from biocide exposure.

KEYWORDS

Outer membrane vesicles, SEM, NanoSAM, Antimicrobial resistance, *Serratia marcescens*

1. INTRODUCTION

Antimicrobial resistance is a crisis with far-reaching impacts in the healthcare, pharmaceutical, agriculture, and food and beverage industries (Pereira and Tagkopoulos, 2019). In addition to the notoriously difficult-to-treat infectious caused by antibiotic resistant microorganisms; we are now facing failures in infection prevention and control measures in healthcare facilities due to the emergence of biocide resistant microorganisms (CDC, 2012; Kovacevic et al., 2015; Langsrud et al., 2003; World Health Organization, 2018). Biocides have active ingredients which are responsible for antimicrobial effects such as Quaternary Ammonium Compounds (QACs), chlorine, ethanol, povidone iodine, and glutaraldehydes to name a few. These active ingredients are responsible for infection prevention and control in healthcare facilities and are used in a multitude of products including disinfectants, cleaning products, antiseptics and hand sanitisers. Research has shown that often biocide and antibiotic resistance evolve simultaneously which can lead to outbreaks (Kim et al., 2018). Therefore, the use of an ineffective biocide can lead to a resistant population within an area of a healthcare facility, often referred to as a “superbug” (Abdelaziz et al., 2019; Laborda et al., 2019; Merchel Piovesan Pereira et al., 2021; Wand et al., 2017). It is in these circumstances that outbreaks occur and lead to multiple hospital-acquired infections (HAIs) for both patients and staff (Bezanson et al., 1983; Mølbak et al., 1999; Nakashima et al., 1987; Weber et al., 2007). When considering the problem of hospital-acquired infections, treatment options are dwindling as antibiotic resistance rises. The lack of new antibiotics in development highlights the need to prevent HAIs instead of treating them (Bragg et al., 2018). To do this, we need to safeguard our current disinfectants and prioritise research into microbial resistance to disinfectants and other biocides.

QACs are used on a large scale in many industries for infection control and to maintain quality standards (Pereira and Tagkopoulos, 2019). Antimicrobial action is brought about by disruption of the cell membrane by the positively charged QACs interacting with the negatively charged bacterial cell walls, penetrating the cell membrane and causing physical and charge disruption (Alkhalifaa et al., 2020; Knauf et al., 2018; Wessels and Ingmer, 2013). QACs integrate into the outer membrane through protein and lipid interactions, resulting in lipid clustering and disruption of the membrane's fluidity (Knauf et

al., 2018; Wessels and Ingmer, 2013). QACs enter the cell envelope by simple adsorption or through porins, leading to damage of the cytoplasmic membrane and an overall loss of cell integrity and content leakage (Ioannou et al., 2007; Pereira and Tagkopoulos, 2019; Wessels and Ingmer, 2013). In addition, benzalkonium chloride (BC), a first generation QAC, has been found to reduce the metabolic activity of cells by 82% and decrease levels of ATP by up to 99% in *Pseudomonas fluorescens* (Barros et al., 2022). However, the precise mechanisms behind these effects are not fully understood (Barros et al., 2022). A fluorescent BC analog, NBD-DDA, showed that tolerance was due to a reduced cellular accumulation of the antimicrobial linked to the efflux pump TolC (Nordholt et al., 2022). This work suggesting that biocidal activity of BC may in full or partly occur from within the cell (Nordholt et al., 2022) and emphasises the paradox of efflux-associated QAC resistance and tolerance. Efflux pumps belonging to *smr* and *qac* families are numerous, and confirmed by multiple studies to confer significantly reduced susceptibility and resistance to QACs (Bjorland et al., 2001; Heir et al., 1999; Jiang et al., 2020; Milani et al., 2021; Paulsen et al., 1996). However, efflux pumps function by reducing intracellular concentrations of antimicrobials (Chitsaz and Brown, 2017; Lomovskaya et al., 2001; Ma et al., 1994; Poole, 2007), therefore, the antimicrobial activity of QACs must work from within cells for antimicrobial susceptibility to be affected by efflux pumps. Recent studies emphasize the potential importance of intracellular targets in the mechanism of action for QACs, indicating that they may play a crucial role in their bactericidal effects (Barros et al., 2022; Nordholt et al., 2022). Microscopy is a useful tool in the study of antimicrobials to uncover their mechanism of action (MOA) and in the discovery of novel resistance mechanisms. NanoScanning Auger Microscopy (NanoSAM) is typically applied in physical science and chemistry disciplines such as materials science and engineering in the study of surface composition, elemental distribution and chemical bonding (Kröner and Hirsch, 2020; Lach et al., 2020). NanoSAM is used on a nanoscale to analyse the elemental composition and surface morphology of materials (Danchuk, 2024). This technique uses an Argon laser to shoot electrons at a sample, causing the atoms in the sample to emit Auger electrons (Danchuk, 2024; Jansen et al., 2013). These electrons have specific energies that correspond to the elements present on the sample surface, thus elucidating the elemental composition of the target site (Danchuk, 2024). NanoSAM can provide

detailed information on the presence of different elements, their relative concentrations and the surface topography and morphology of the sample with high spatial resolution(Jansen et al., 2013).

There are numerous applications for nanotechnology and nanomaterials where characterisation of nanoparticles can be done in terms of morphology, interactions and general composition of materials (Potter et al., 2018; Yulaev et al., 2018). However, this powerful tool can be used in many other disciplines including antimicrobial susceptibility studies for microorganisms. Previous work on *Staphylococcus aureus* used SAM technology to research the effects of antimicrobials on cells (Jansen et al., 2013). This bacterium was exposed to Didecyldimethylammonium chloride (DDAC) a QAC disinfectant to ascertain any morphological differences and an attempt to localise the QAC compound within the cell (Jansen et al., 2013). At extremely high levels of the biocide DDAC, major morphological changes occurred, with an increase in cell nodules together with cell leakage (Jansen et al., 2013). This work aims to build on this previous study by examining a highly resistant *Serratia* sp. isolate compared to the closest related type strain *Serratia marcescens* subsp. *marcescens*, in terms of morphological changes that occur after exposure to two different QAC-based compounds, benzalkonium chloride (BC) and Virukill® (a DDAC-based product). In addition, atomic percentage analysis will be conducted in an attempt to localise the QAC-molecules either within or outside the bacterial cells. Although this is not the typical application of NanoSAM technology, this work shows that it could be an invaluable resource in the fight against antimicrobial resistance as we elucidate more on the mode of action of antimicrobials and the coupled resistance mechanisms.

2. MATERIALS AND METHODS

2.1. Microorganisms

Two bacteria used in this study were *Serratia* sp. HRI obtained from the University of the Free State, South Africa culture collection; and the closest related type strain, *Serratia marcescens* subsp. *marcescens* ATCC 13880, from the American Type Culture Collection (Mc Carlie et al., 2020). Minimal inhibitory concentrations (MICs) for both bacteria against DDAC, BC and Virukill®™ were previously determined and published (Mc Carlie et al., 2020).

2.2. Disinfectant treatment

Benzalkonium chloride (BC) from Sigma-Aldrich ($\geq 50\%$ in water) and Virukill®™ (Didecylammonium Chloride 120 g/L) were used. Short-term (20 minute) exposure was analysed with NanoSAM at sub-MIC concentrations of 0.98mg/ml BC and 120 mg/l Virukill®. Long-term exposure (24 hours) analysed with SEM was done at concentrations of 9.8mg/ml BC and 240 mg/l Virukill® for *Serratia* sp. HRI and 0.098mg/ml BC and 12 mg/l Virukill® for *Serratia marcescens* ATCC.

2.3. Cell morphology and atomic percentage: Cell cultivation for NanoSAM

Inoculum of *Serratia* sp. HRI was prepared by transferring a single colony from 24h agar plates to 25mL LB medium (10 g/L Merck Tryptone, 10 g/L Merck sodium chloride, 5 g/L Merck yeast extract) in 250mL Erlenmeyer flasks and incubated at 37°C for 16h. From this pre-inoculum, 2.5mL was added into 500mL Erlenmeyer flasks containing LB media supplemented with one of the three QAC-based disinfectants (benzalkonium chloride, Virukill®™, and DDAC) and incubated at 37°C shaking at 250 r min⁻¹. Shake cultivation was chosen for this application to hinder biofilm formation to enhance cell morphology visualisation and atomic percentage analysis. After 90 minutes of cultivation, either water (Untreated samples), or disinfectant (treated samples) was added and a contact time of 20 minutes elapsed before cells were harvested by centrifugation at 5000 x g for 5 minutes at 4°C.

2.4. Biofilm formation: Cell cultivation for SEM

Inocula of *Serratia* sp. HRI and *S. marcescens* ATCC 13880 were prepared by transferring a single colony from 24 h agar plates to 10mL LB pre-inoculum medium (10 g/L Merck Tryptone, 10 g/L Merck sodium chloride, 5 g/L Merck yeast extract). After cultivation overnight, a 10% inoculum (approximately 3.0×10^8 cells) was added to fresh media containing water (untreated), benzalkonium chloride, or Virukill® disinfectants at a concentration of 50% of the 24h MIC. The treated cultures were incubated at 37°C in stationary test tubes for 24 hours to prevent hindrance of biofilm formation before fixation.

2.5. Microscopy preparation

Material was fixed in 0.1M (pH 7.0) sodium phosphate-buffered glutardialdehyde (3%) for at least 3 hours, followed by 1 hour fixation in similarly buffered osmium tetroxide (1%). The material was collected on 0.2µm polycarbonate membrane filters and dehydrated in a graded ethanol series (50%, 70% and 95% for 20 minutes in each phase followed by two changes of 100% for 1 hour in each phase). The material was dried using a critical point dryer (Tousimis, Maryland, U.S.A.). After drying, material was mounted on stubs (Cambridge pin type, 10mm) using double sided carbon tape and gold coated ($\pm$ 10 nm) with a Bio-Rad sputter coater (United Kingdom) for NanoSAM preparation.

The manuscript does not contain experiments using animals.

The manuscript does not contain human studies.

3. RESULTS

3.1. *Serratia* sp. HRI short-term exposure (20-minutes)

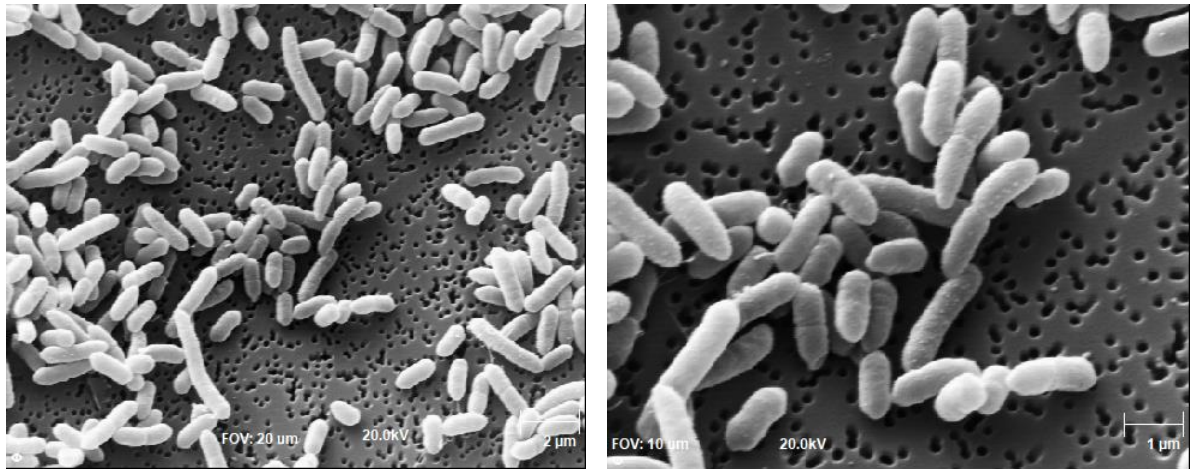


Figure 1 Untreated *Serratia* sp. HRI visualised with NanoSAM technology.

Untreated *Serratia* sp. HRI cells, in Figure 1, were found to have typical cellular morphology for the *Serratia* species, as straight rods with rounded ends. Untreated cells lacked a biofilm and a few cell nodules were observed on the surface. Different cell lengths are seen as cells are in varying stages of division with no cell damage present.

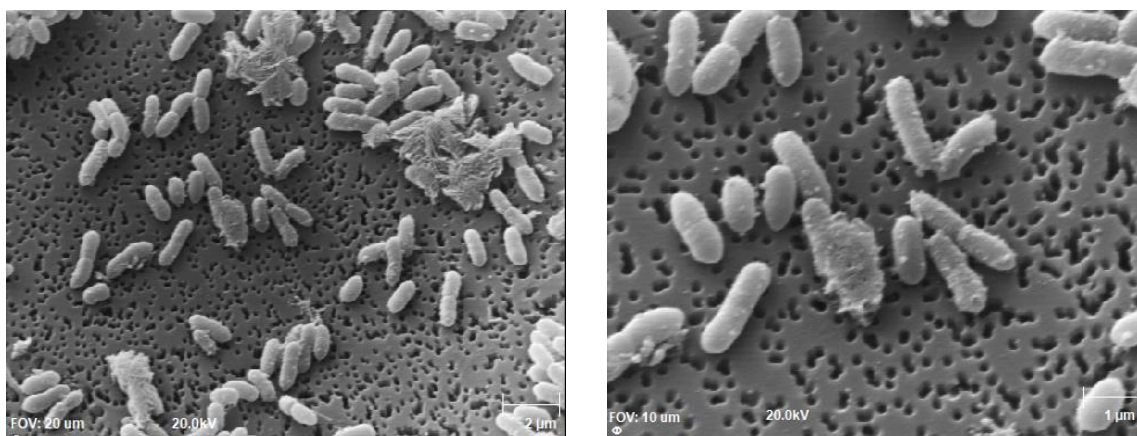




Figure 2 *Serratia* sp. HRI after short-term treatment with benzalkonium chloride at 0.98 mg/ml.

After treatment with sub-MIC levels of BC biocide, depicted in figure 2, cell morphology changed drastically. An increase in cell nodules was observed for all cells, together with a rougher cell surface. Cell leakage was observed from holes in the cell wall and swollen cells suggest a breakdown in cell integrity and structure after treatment. Cell leakage and aggregated cytoplasm depict evidence of cell lysis after treatment with BC.

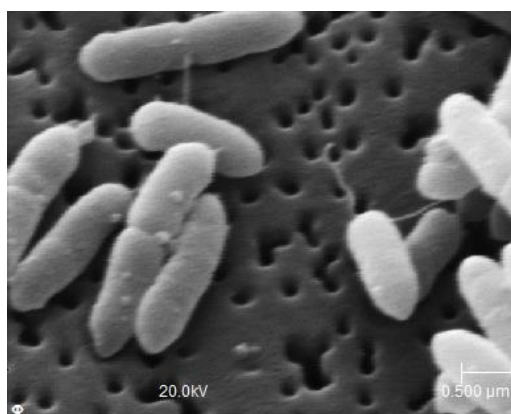
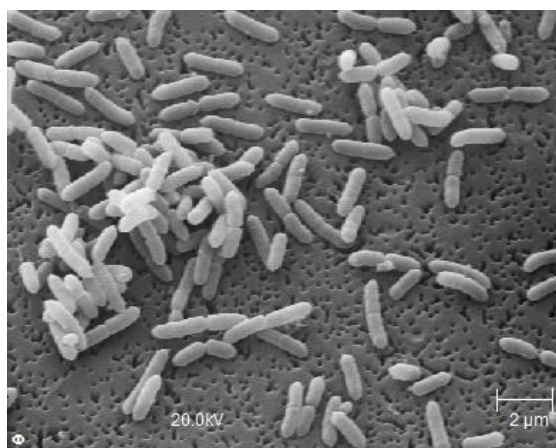


Figure 3 *Serratia* sp. HRI after short-term treatment with Virukill® at 120 mg/l.

Treatment with Virukill® shown in figure 3, led to no major observable changes in cell texture or morphology compared to the untreated samples. Typical cellular morphology for the *Serratia* species, was observed and cells lacked a biofilm. However, an increased amount of cell nodules was seen on the cellular surface. Varying cell lengths were observed as cells are in different stages of division with little to no cell damage after treatment with sub-MIC levels of Virukill™.

A comparison of the BC- and Virukill-treated *Serratia* sp. HRI elucidated major morphological changes between the two treatments. Treatment with Virukill™ revealed an increase in cell nodules but very little changes in cell morphology, resembling the untreated cells. Whereas, treatment with BC altered cell morphology drastically with an even greater increase in the amount of cell nodules, a rough cell surface, loss of cell structure and integrity and evidence of cytoplasm leakage. These results confirm part of the mechanism of action of QAC compound BC as disruption of the cell membrane. However, these results also reveal differences between the products from different generations of QACs, BC representing older generation QACs and Virukill™ representing newer generations of QACs.

Cell leakage elucidated in Figure 2, depicts disruption of the cell membrane by the QAC disinfectant, however it is still unclear whether these compounds work from within the cell or from outside the cell. Therefore, atomic percentage analysis was conducted to examine if an increased level of nitrogen could be detected within cells compared to untreated cell populations, representing the high levels of QAC compounds used for treatment.

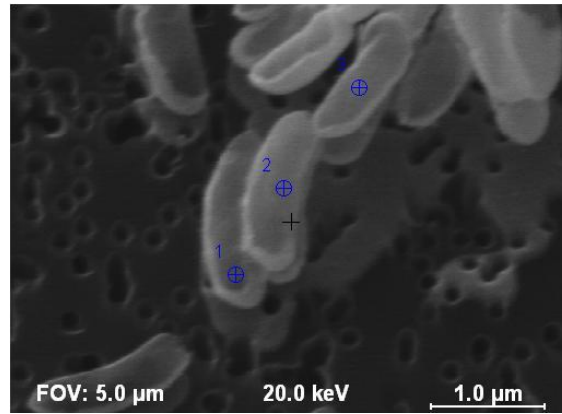


Figure 4 Atomic percentage analysis. Atomic layers were removed from the cells with an Argon (Ar^+) ion gun and points within the cells were chosen for analysis.

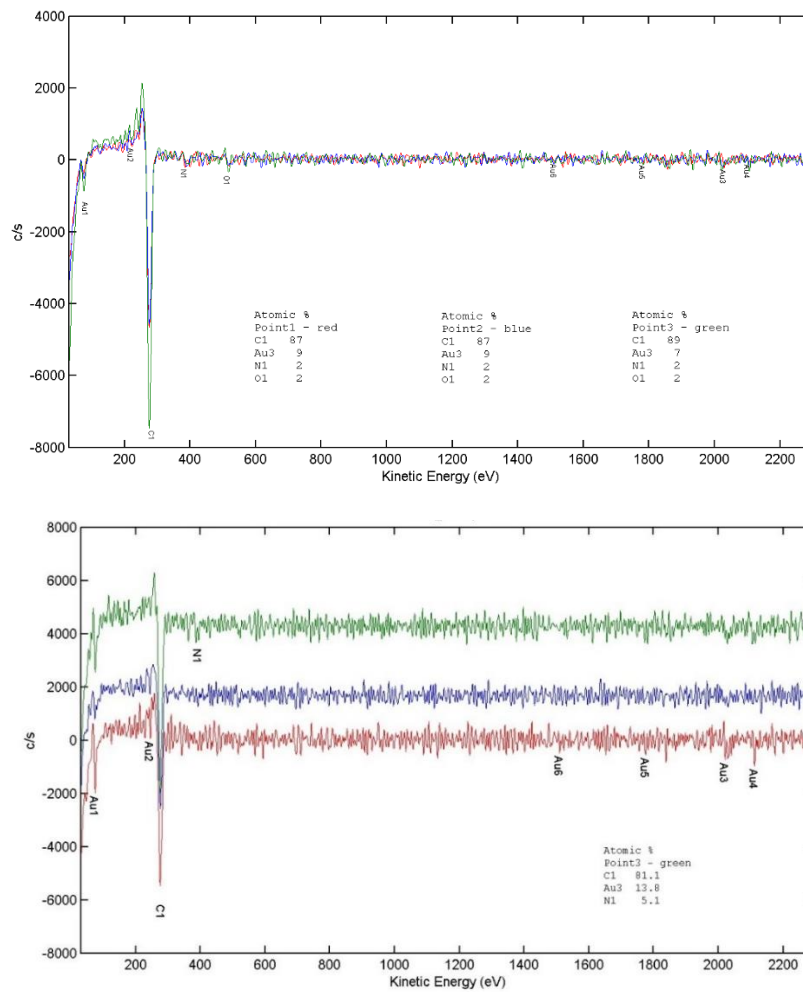


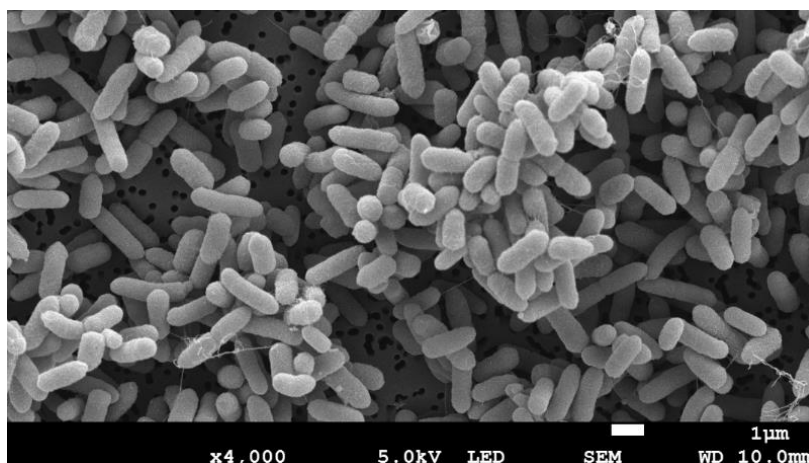
Figure 5 Atomic percentage analysis of untreated and treated cells of *Serratia* sp. HRI.

Cells were sputtered with the Ar⁺ ions layer by layer to remove the gold coating and cut into the cells. Atomic percentage analysis (shown in Figures 4 and 5) commenced when the typical “boat-like” structure was seen indicating the removal of the cell membrane (indicated by a light cell surface due to external gold coating). The inside of the cells have been reached when a dark shadow appears within the cells, as the light cell surface (due to gold coating) has been removed and sputter depth indicated mid-cell depth inferring from cell size., Pin-points indicate where analysis was done and the resultant atomic percentage graphs generated.

No major cell morphology changes were observed before sputtering commenced, rigid cell structures during cell sputtering indicated a good level of fixation on cells. Atomic percentage analysis depicted in Figure 5 indicated most prevalent atomic elements within the target area, common elemental results included carbon, nitrogen and oxygen as expected of bacterial cells, together with gold due to coating during SEM stub preparation.

3.2. Long-term exposure (24 hours)

3.2.1. *Serratia* sp. HRI Untreated



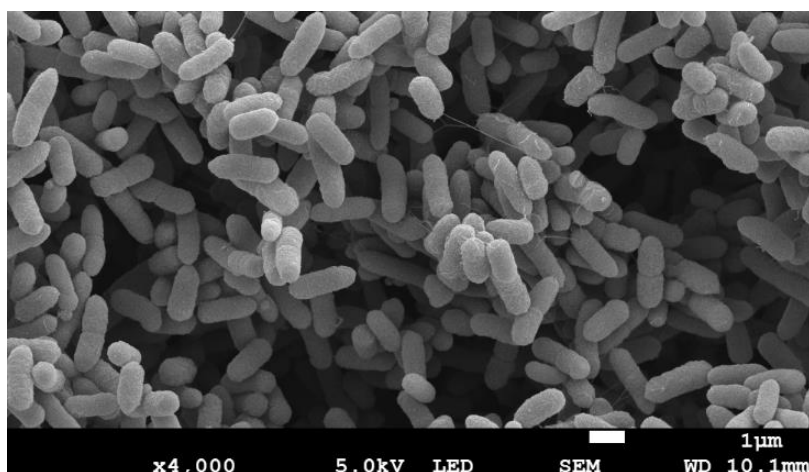
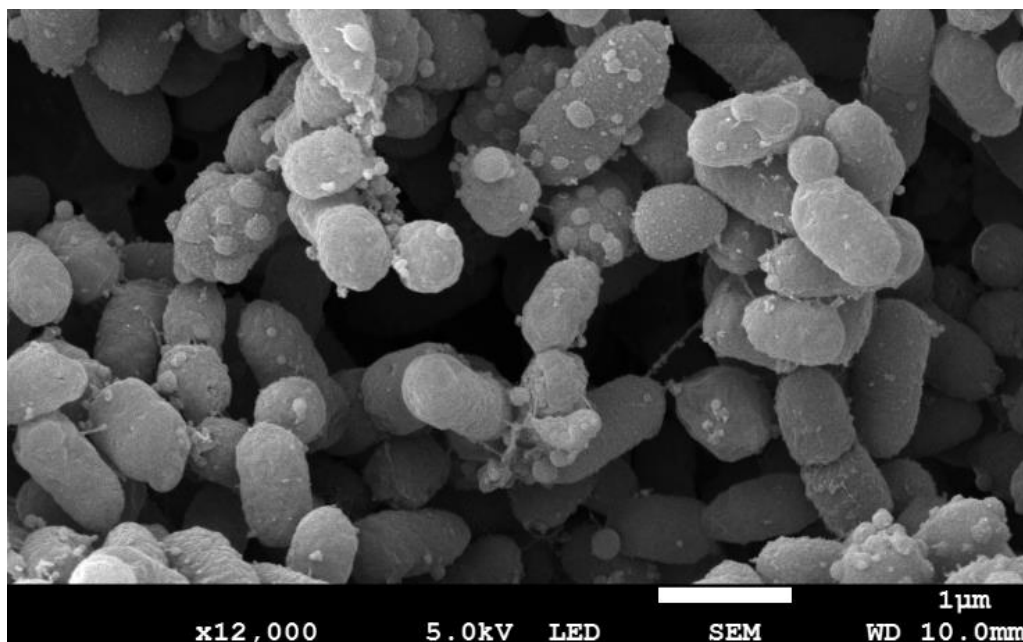


Figure 6 *Serratia* sp. HRI after 24-hour incubation period.

In the 24-hour untreated *Serratia* sp. HRI sample shown in figure 6, the typical cellular morphology for the *Serratia* species, was observed as straight rods with rounded ends. In the sample containing untreated cells, a few cell nodules could be found within the sample on the cell surface and no biofilm was formed evident by a lack of biofilm matrix and elements within the sample.

3.2.2. *Serratia* sp. HRI Benzalkonium chloride treatment



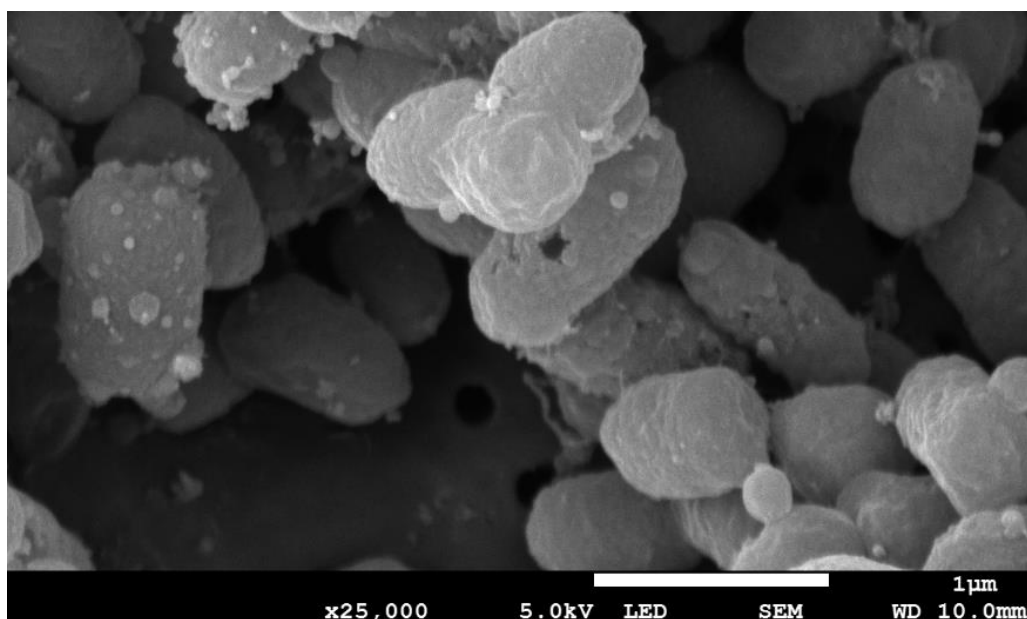
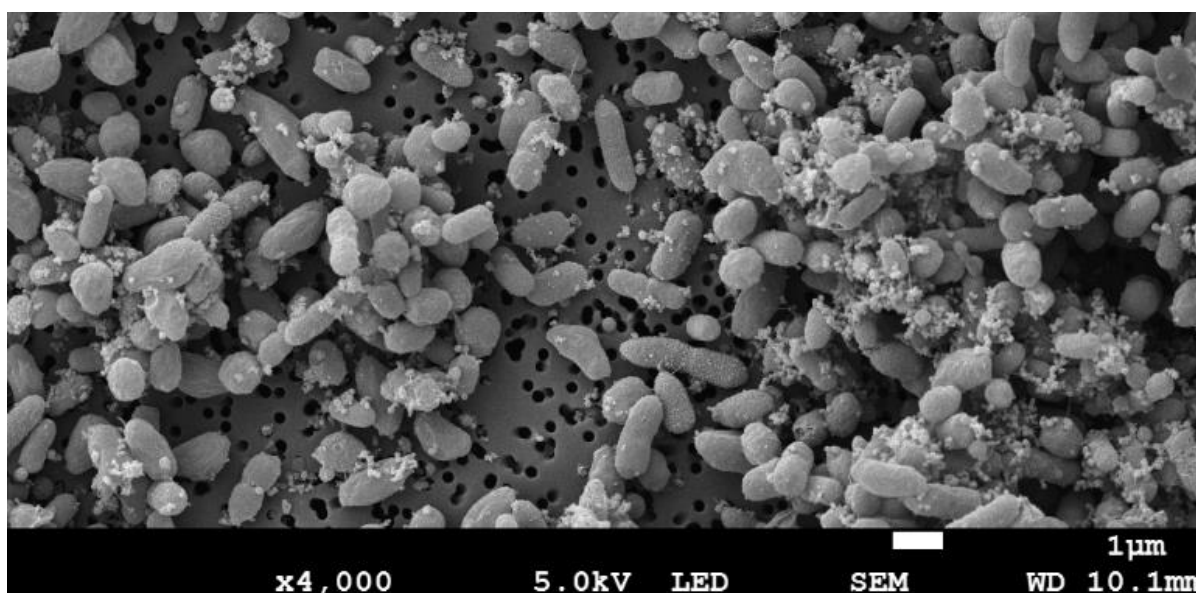


Figure 7 *Serratia* sp. HRI after 24-hour treatment with benzalkonium chloride at 9.8 mg/ml.

After a 24-hour exposure to BC, the resistant isolate, shown in figure 7, had a major increase in cellular surface nodules and damage to the cell wall evident by misshapen cells, holes in the cell wall and lysed cells. No biofilm formation occurred during the 24-hour treatment period.

3.2.3. *Serratia* sp. HRI Virukill® treatment



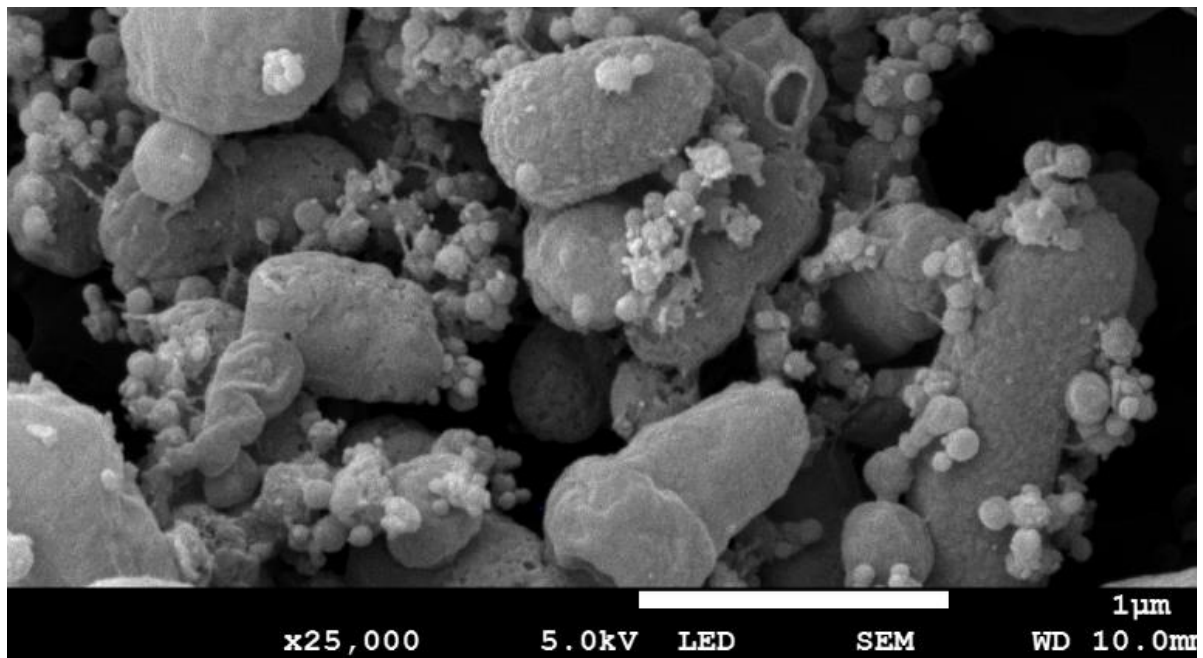
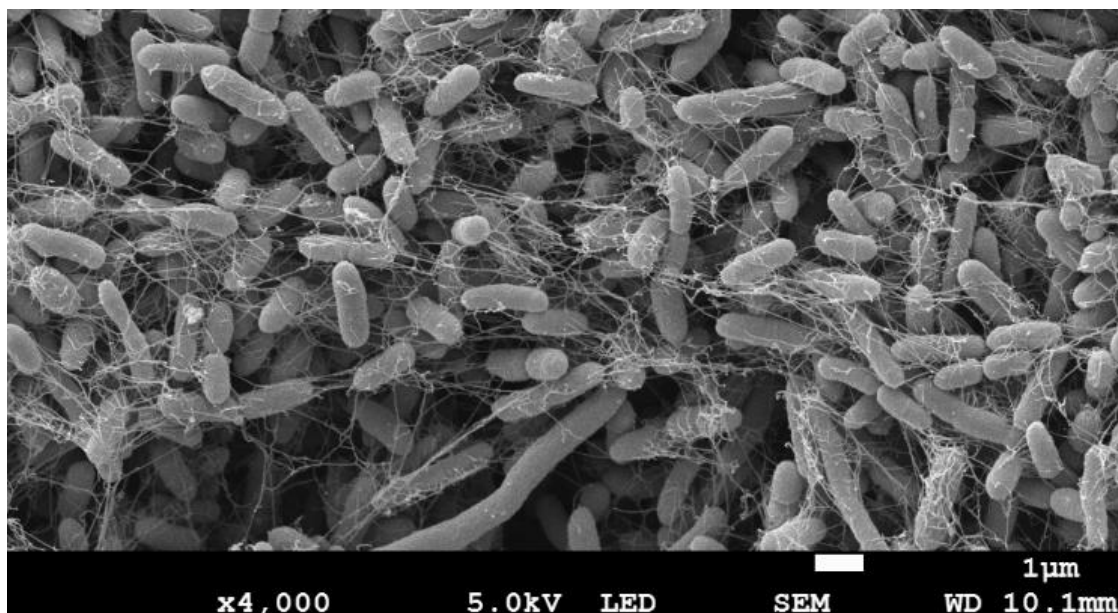


Figure 8 *Serratia* sp. HRI after 24-hour treatment with Virukill® at 240 mg/l.

A 24-hour exposure to Virukill® for the resistant isolate, shown in figure 8, lead to a significant increase in nodules on the cell surface together with observable damage to the cell walls of cells. Additionally, after 24-hour exposure to the biocide, no biofilm formation occurred.

3.2.4. *Serratia marcescens* ATCC 13880 Untreated



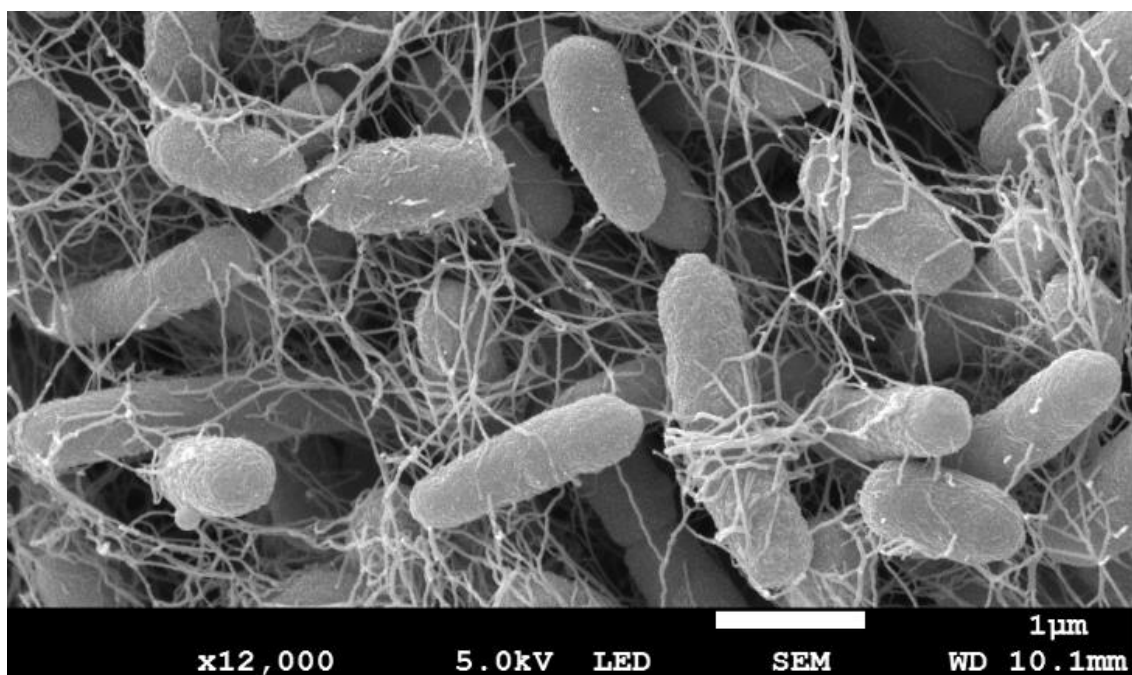
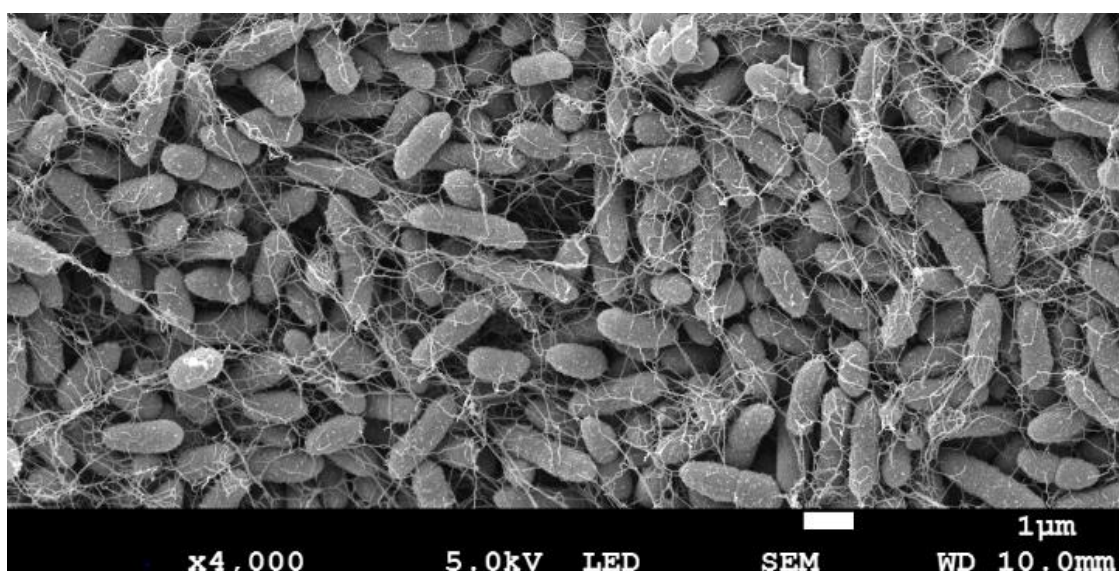


Figure 9 *Serratia marcescens* ATCC after 24-hour incubation period.

Untreated cells of the type strain, *Serratia marcescens* subsp. *marcescens* ATCC 13880, can be observed in figure 9. After 24-hours of incubation, a thick filamentous biofilm had formed and a textured cell surface was noted.

3.2.5. *Serratia marcescens* ATCC 13880 Benzalkonium chloride



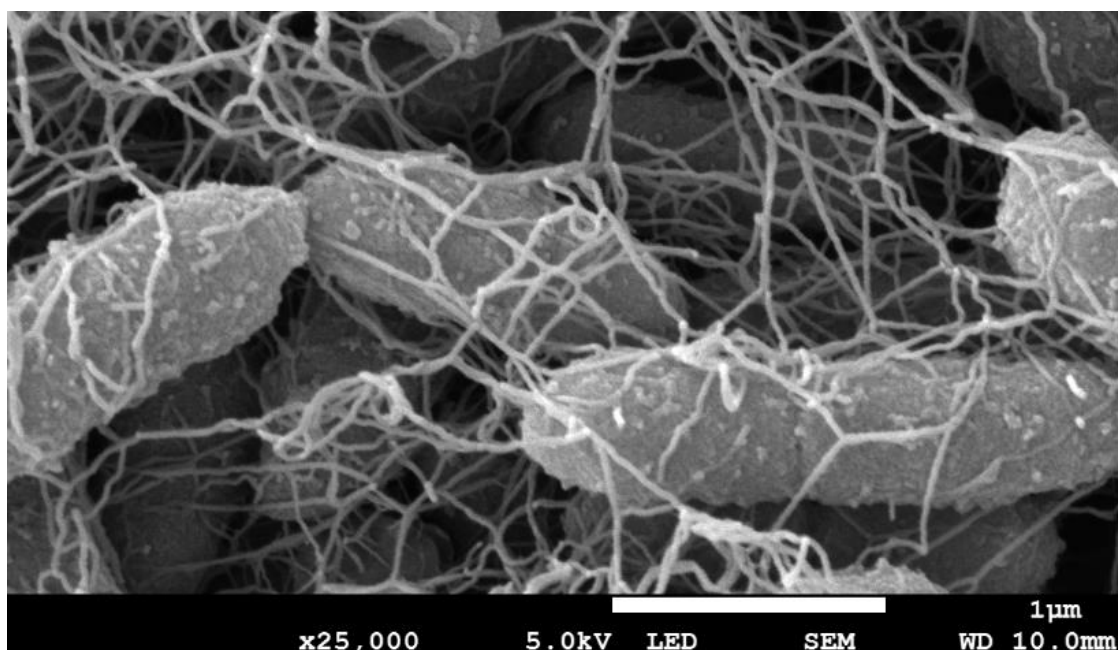
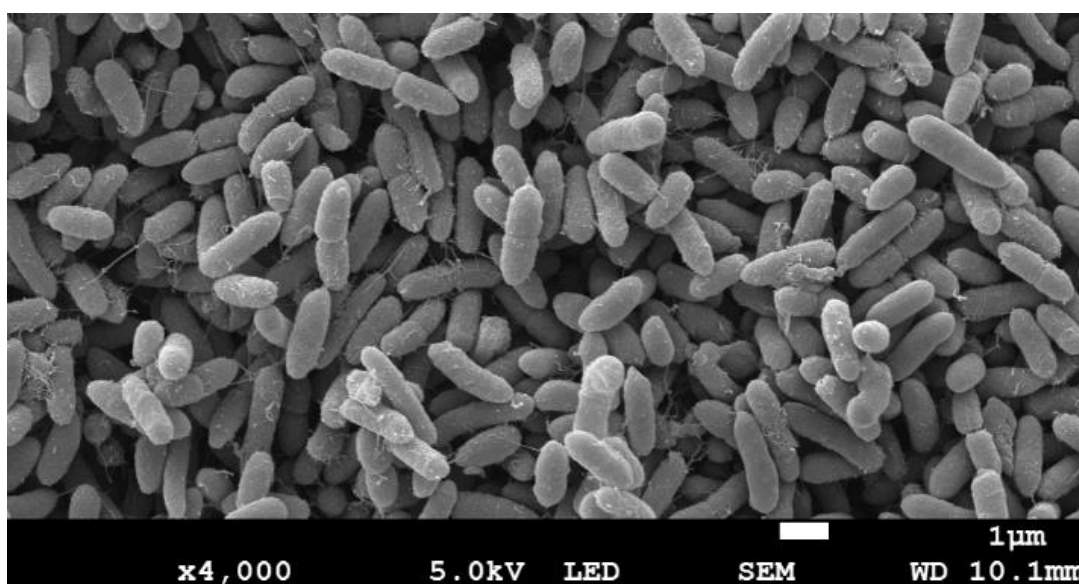


Figure 10 *Serratia marcescens* ATCC after 24-hour treatment with benzalkonium chloride at 0.098 mg/ml.

After 24-hour exposure to BC for the type strain, depicted in figure 10, biofilm formation was unchanged compared to the untreated samples and no major cell morphology changes were observed.

3.2.6. *Serratia marcescens* ATCC 13880 Virukill®



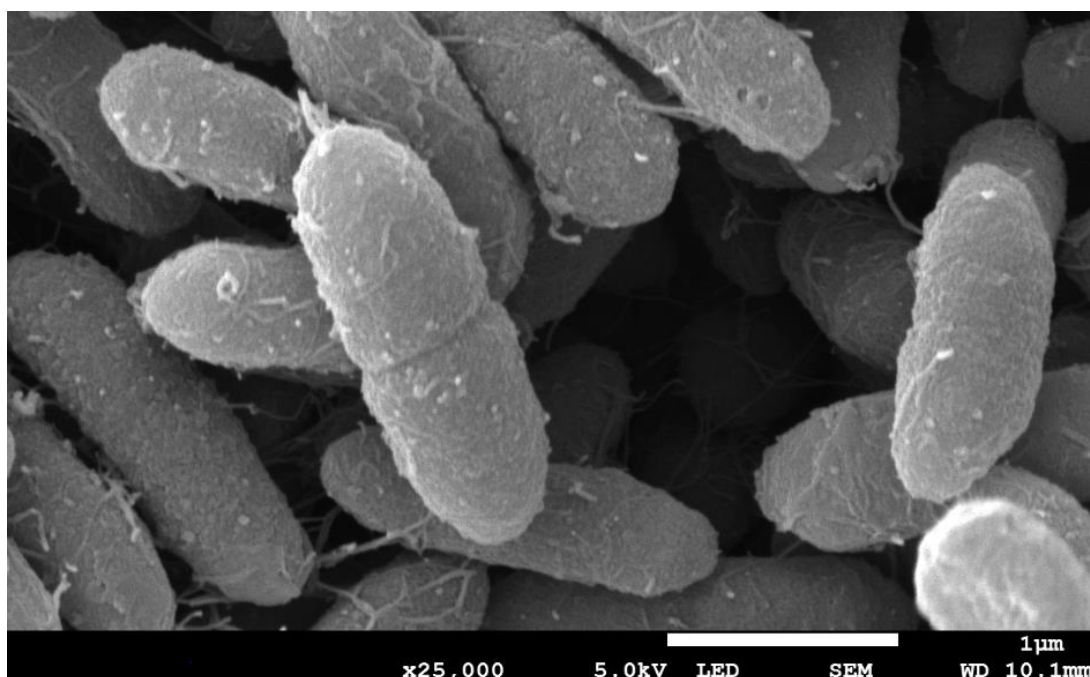


Figure 11 *Serratia marcescens* ATCC after 24-hour exposure to Virukill® at 12mg/l.

Virukill®-treated cells belonging to the type strain, in figure 11, showed no biofilm formation after 24-hours of exposure. This is in contrast to both the untreated and BC-treated samples where thick filamentous biofilms were observed. Cell wall damage was seen together with cell leakage as a result of the Virukill® treatment.

4. DISCUSSION

Microscopy techniques, NanoSAM and SEM, revealed cell morphology changes after a short (20 minute) and long (24-hour) contact time with QAC disinfectants benzalkonium chloride and Virukill®.

Serratia marcescens ATCC exposed to the QAC product Virukill® showed hindrance of biofilm formation after 24-hour exposure. The product Virukill® has the ability to interfere with biofilm formation could have major impacts on the antimicrobial industry and direct future formulations of biocides.

The resistant isolate *Serratia* sp. HRI, formed no biofilm even after 24-hours of exposure to both QAC disinfectants. This suggests that the extraordinary high levels of resistance to QACs by this isolate is not due to biofilm formation. Furthermore, this suggests that biofilm formation is not a true resistance mechanism against QAC biocides. This hypothesis is supported by recent transcriptomic analysis of

bacteria exposed to QAC disinfectants where biofilm-associated genes were not found to be differentially expressed after exposure to QACs (McCarlie et al., 2023). Unexpectedly, what was observed after the resistant isolate was exposed to BC was numerous nodules on the cell surface after exposure to BC and Virukill®. These nodules on the cell surface could be outer membrane vesicles (OMVs) which have been found to decrease susceptibility to antibiotics (S. W. Kim et al., 2018; Roszkowiak et al., 2019). OMVs act as a physical barrier to prevent antibiotics from reaching the cell wall and cellular components beneath (Roszkowiak et al., 2019). Additionally, OMVs carry antibiotic-degrading enzymes such as β -lactamases which are capable of breaking down antibiotics and therefore protect microbial cells (S. W. Kim et al., 2018). This work suggests that a similar resistance mechanism may be at play in reducing susceptibility to biocides, although further analysis is required.

When analysing the atomic percentage results from the NanoSAM results, a significant difference was found in the atomic percentage of nitrogen (N) between untreated and treated cells; untreated cells scored an average nitrogen percentage of 2.69% and treated samples scored an average nitrogen percentage of 4.13%, a significant difference between the two with 99% confidence level. The increased level of N within the cell could be due to an accumulation of QACs within the cell which contain nitrogen as part of their molecular structure. Conversely, this increase in intracellular N could also be part of the response of the cell to the QAC attack and not due to the QAC molecules themselves. Recent research with fluorescently labelled QAC compounds found that a reduction in the intracellular accumulation by efflux of BC was responsible for BC tolerance in *Escherichia coli* (Nordholt et al., 2022). These findings support the hypothesis posed here that the QAC biocides do accumulate within the cell. As a result, QAC biocides most likely work from inside the cell instead of outside, as is our current understanding of MOA of QAC biocides. Furthermore, this hypothesis is supported by the undeniably major impact efflux pumps have on QAC susceptibility which reduces intracellular concentrations of antimicrobials (Fraud et al., 2008; Mavri and Mozina, 2012; Short et al., 2021; Wand et al., 2022).

5. CONCLUSIONS

The results presented reveal the value of microscopy techniques in the study of antimicrobial susceptibility and resistance. These results challenge current hypotheses on the mechanism of action of QAC biocides and resistance mechanisms employed by microorganisms. This work builds on the evidence that QACs work from inside the cell and not outside as the current hypothesis suggests. Furthermore, the evidence gathered in this work and in literature suggests biofilm formation is not a resistance mechanism against QAC biocides. Instead, resistant bacteria produce OMVs to reduce susceptibility to biocides and antibiotics.

6. ACKNOWLEDGEMENTS

The authors would like to acknowledge the centre of microscopy at University of the Free State.

7. FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

8. CONFLICT OF INTEREST

Authors have no conflict of interest to disclose.

9. CRediT

S. J. McCarlie: Conceptualisation (lead), data curation (lead), Formal Analysis (lead), Investigation, Methodology, validation, writing, (original draft); **E. Coetsee:** Methodology, Resources, Software, Visualisation, Writing – Review & Editing; **H. Grobler:** Investigation, methodology, software, Viualisation, Writing – Review & Editing; **R. R. Bragg:** Funding Acquisition, Project Administration, Supervision, Writing – Review & Editing.

10. ETHICAL APPROVAL

Environment & Biosafety Research Ethics Committee ethical clearance number: UFS-ESD2022/0125/22

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3.3. Chapter 3 Conclusions

When considering the microscopy results presented in this chapter, much is illuminated regarding the growth analysis in the second chapter. The significantly higher levels of atomic nitrogen within treated cells suggests that the QAC compounds may be located within the cells. This would explain the drop in OD observed within the first 30 minutes of growth for *Serratia* sp. HRI as the disinfectant compounds exit the media suspension and enter cells. The current hypothesis amongst literature is that QACs cause cell damage from the outside of cells (Alkhalifaa et al., 2020; Obłąk et al., 2021; Wessels and Ingmer, 2013), however evidence in chapters 2 and 3 suggests that this hypothesis is false, and that QAC disinfectants may work from inside cells instead. Furthermore, results from the SEM imaging suggest biofilm formation does not play a role in the extraordinary resistant phenotype of *Serratia* sp. HRI. This is contradictory to our current beliefs that biofilm formation is an important resistance mechanism (Knauf et al., 2018; Milani et al., 2021; Pereira and Tagkopoulos, 2019). Interestingly, the lack of biofilm formation of *Serratia* sp. HRI concurs with literature in Chapter 1 where no differential expression of biofilm-associated genes was observed for bacteria exposed to benzalkonium chloride (McCarlie et al., 2023). This proposes that biofilms do indeed reduce susceptibility in a microbial community, if they are established before exposure. The act of exposure to benzalkonium chloride and other QACs, does not trigger biofilm formation as it is not a resistance mechanism but a survival advantage if already established.

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Chapter 4: DGE analysis of *Serratia* sp. HRI at sub-MIC levels of BC at various time points to screen for novel biocide resistance genes

4.1. Introduction

Previous work presented in this study has focused on phenotypic changes in response to QAC exposure. Chapter 4 of this dissertation marks the shift to molecular analysis of *Serratia* sp. HRI when exposed to QAC disinfectants. The hypothesis is that this highly resistant isolate differentially expresses genes after exposure to QAC disinfectants to activate resistance mechanisms. This chapter aims to screen for novel resistance genes and generate a list of potential novel resistance genes. Through RNA-Seq, lists of the most differentially expressed genes will be generated to represent targets for future studies to uncover novel resistance genes. From this list, a target gene will be chosen for this study, where differential expression will be confirmed using quantitative real-time PCR.

This experimental chapter has been published and is entitled “Differential Expression Analysis Reveals Possible New Quaternary Ammonium Compound Resistance Gene in Highly Resistant *Serratia* sp. HRI” in the journal *Microorganisms*. This is a peer-reviewed, accredited scientific journal. As the first author, I researched the topic, designed the methodology, conducted all experiments, wrote, and compiled the article. The co-authors of this article are my two promoters, Prof. R. R. Bragg and Dr. C. E. Boucher-van Jaarsveld, who guided methodology design, data analysis and manuscript editing. This published original research paper is presented as the complete fourth chapter of this dissertation.



Article

Differential Expression Analysis Reveals Possible New Quaternary Ammonium Compound Resistance Gene in Highly Resistant *Serratia* sp. HRI

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Abstract: During the COVID-19 pandemic, the surge in disinfectant use emphasised their pivotal role in infection control. While the majority of antimicrobial resistance research focuses on antibiotics, resistance to biocides, which are present in disinfectants and sanitisers, is escalating. *Serratia* sp. HRI is a highly resistant isolate, and through the study of this organism, the molecular mechanisms of resistance may be uncovered. *Serratia* sp. HRI was treated with the disinfectant benzalkonium chloride in preparation for RNA sequencing. Through mining of the RNA-Seq differential expression data, an uncharacterised Major Facilitator Superfamily (MFS) efflux pump gene was found to be up-regulated at least four-fold at four different time points of exposure. Real-time PCR revealed this uncharacterised MFS efflux gene was up-regulated after exposure to benzalkonium chloride and two additional disinfectants, didecyltrimethylammonium chloride (DDAC) and Virukill™. Additionally, expression of this gene was found to be higher at 20 min versus 90 min of exposure, indicating that the up-regulation of this gene is an initial response to biocide treatment that decreases over time. This suggests that MFS efflux pumps may be an initial survival mechanism for microorganisms, allowing time for longer-term resistance mechanisms. This work puts forward a novel biocide resistance gene that could have a major impact on biocide susceptibility and resistance.

Keywords: biocides; antimicrobial resistance; MFS efflux; RNA-Seq; qPCR



Citation: McCarlie, S.; Boucher-van Jaarsveld, C.; Bragg, R. Differential Expression Analysis Reveals Possible New Quaternary Ammonium Compound Resistance Gene in Highly Resistant *Serratia* sp. HRI.

Microorganisms **2024**, *12*, 1891.

<https://doi.org/10.3390/microorganisms12091891>

Academic Editor: Victoria Birlutiu

Received: 26 August 2024

Revised: 5 September 2024

Accepted: 8 September 2024

Published: 13 September

2024



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1. Introduction

Antimicrobial resistance is a well-known issue, although the vast majority of research focuses on resistance to antibiotics. A lesser-researched form of antimicrobial resistance is resistance to biocides, which form the active ingredients in disinfectants, antiseptics, and hand sanitisers. During the COVID-19 pandemic, our reliance on biocides was highlighted through extensive use of disinfectants and sanitisers for infection prevention. These products are not only crucial in healthcare for infection control but are important in agriculture for disease protection and in the food and beverage industry to maintain hygiene protocols, thus safeguarding food security worldwide [1].

During the COVID-19 pandemic, the United States Environmental Protection Agency (EPA) published a list of approved disinfectants for use against SARS-CoV-2 [2]. Disinfectant products containing quaternary ammonium compounds (QACs) as the active ingredient were the majority of disinfectants approved [2]. Within this group, benzalkonium chloride (BC) and didecyltrimethylammonium chloride (DDAC) were the most common active ingredients [2]. Alarming, overuse and improper applications of disinfectants during the pandemic increased the selective pressure on microorganisms and has accelerated the development of reduced susceptibility and resistance to biocides [3–6]. In addition, this resistance is not specific, as research has shown that if a microorganism develops resistance to biocides, antibiotic resistance can develop simultaneously without prior exposure [7,8].

New advances in sequencing technology and data analysis, coupled with broader databases, have revealed the impact of molecular mechanisms of antimicrobial resistance [9–11]. This has allowed for the discovery of novel resistance genes and mechanisms, and for the surveillance of antimicrobial determinants [11–14]. Together, this allows for further insight into the epidemiology of resistance and elucidation of how resistant populations develop. One such microorganism is *Serratia* sp. HRI, an isolate with high resistance capabilities to several active ingredients used in disinfectants and other biocides [15,16]. Genome sequence analysis revealed *Serratia marcescens* to be the closest related species [16], which is a notorious pathogen responsible for a number of nosocomial outbreaks in neonatal and paediatric facilities [17–20]. The study of this isolate is a unique opportunity that may reveal novel molecular mechanisms that confer the highly resistant phenotype. Three different generations of QAC products were used in this study, as these products are used globally in medical facilities and agriculture for infection control. This work used transcriptomics in the form of RNA-Seq to screen for exceedingly differentially expressed genes after exposure to BC at various time points. After mining the transcriptomic data, lists of novel targets for QAC-resistance genes were generated. One gene was singled out in this work and real-time PCR was performed to further analyse the differential expression of this gene after exposure to two additional QAC disinfectants: DDAC and VirukillTM. This work has generated lists of possible novel QAC-resistance genes for further study and confirmed the significant differential expression and role played by MFS-efflux pumps in QAC resistance.

2. Materials and Methods

2.1. Bacterial Strains

This study focused on the highly resistant isolate *Serratia* sp. HRI [16] obtained from the culture collection of the University of the Free State. This strain was part of a batch of mixed environmental samples and was subsequently found in its pure form contaminating a bottle of QAC disinfectant, where it was isolated [16].

2.2. Disinfectants and Conditions

Benzalkonium chloride (BC) was obtained from Sigma-Aldrich (St. Louis, MO, USA) ($\geq 50\%$ in water), while didecyldimethylammonium chloride (DDAC) (80% UNIQUT) and VirukillTM (DDAC 120 g/L) were both obtained from ICA International Chemicals (Plankenbrug, South Africa). The minimal inhibitory concentration (MIC) levels of these disinfectants for *Serratia* sp. were previously reported [16], and in this study, all the disinfectants were used at sub-MIC levels as follows: BC at a sub-MIC level of 9 800 mg/L, DDAC at 76.62 mg/L, and VirukillTM at 1 200 mg/L.

2.3. Bacterial Cultivation

The *Serratia* sp. HRI was prepared as described in previous growth kinetics studies [15]. After 60 min, a sub-MIC level of benzalkonium chloride was added to the inoculum (except the untreated control samples) and a contact time of 10, 20, 30, or 90 min was allowed, as depicted in Table 1, before the cell pellets were immediately treated with Invitrogen RNAlater Stabilization Solution to store and stabilise the RNA within the cells before RNA extraction commenced (Invitrogen, Carlsbad, CA, USA).

Table 1. Experimental conditions and abbreviated sample names used in this study.

Condition and Timepoint of RNA Extraction	RNA-Seq Sample Name Abbreviation
Untreated/Control 10 min	+10
Untreated/Control 20 min	+20
Untreated/Control 30 min	+30
Untreated/Control 90 min	+90
Benzalkonium chloride exposure for 10 min	D10
Benzalkonium chloride exposure for 20 min	D20
Benzalkonium chloride exposure for 30 min	D30
Benzalkonium chloride exposure for 90 min	D90

2.4. RNA Extraction and Quality Control

RNA extraction was performed on fresh cell pellets using the RNeasy mini kit (Qi-agen, Hilden, Germany) according to the manufacturer's instructions. An additional on-column DNase digestion was performed to eliminate possible genomic DNA (gDNA) contamination using the ZymoResearch DNase I set as per the manufacturer's instructions (ZymoResearch, Irvine, CA, USA), and the DNA digestion was confirmed by agarose gel electrophoresis. The total RNA integrity and purity assessments were performed using the 2100 Agilent Bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. All the samples had to adhere to an RIN value of ≥ 7.5 . The purity (A260/A280 ratios) and concentrations were determined with the NanoDrop 1000 spectrophotometer and the concentration of the RNA samples was measured using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Once all the samples had passed the QC based on the RNA integrity, concentration and purity, the samples were sent to the Centre for Proteomic and Genomic Research for sequencing (Cape Town, South Africa) and were prepared as described below. RNA extracts were used for both the RNA-Seq and conversion to cDNA for real-time downstream validation.

The total RNA for all the samples was precipitated by the addition of sodium acetate (0.3 M) and 3 volumes of ice-cold absolute ethanol. The RNA was allowed to precipitate at -80°C overnight. The RNA was pelleted by centrifugation at $13,000\times g$ for 10 min. The RNA pellets were washed twice with ice-cold 70% ethanol and centrifuged at $13,000\times g$ for 10 min. The ethanol was removed and the pellets were dried at room temperature and then resuspended in nuclease-free water. The RNA was evaluated for purity using the NanoDrop 8000 spectrophotometer, for quantity using the Qubit RNA BR Assay Kit, and for integrity using the Agilent Bioanalyzer RNA 6000 Nano Kit. Bacterial ribosomal RNA was depleted using the Invitrogen RiboMinus Bacteria module according to the manufacturer's instructions, using 5 μg of each sample. The resulting ribosomal-depleted RNA was concentrated using the Invitrogen RiboMinus Concentration Module.

2.5. RNA-Seq and QC

The ribosomal-depleted RNA was used for the Illumina library preparation using the NEXTflex Rapid Directional RNA-Seq Kit according to the manufacturer's instructions. The ribosomal-depleted RNA was fragmented and converted to single-stranded cDNA, followed by second-strand synthesis using dUTP. The cDNA was purified by using Agencourt AMPure XP magnetic beads, followed by adenylation and adapter ligation. The adapter-ligated cDNA was purified by using Agencourt AMPure XP magnetic beads, then treated

with Uracil DNA Glycosylase to maintain the strand information, and finally, amplified by PCR for 15 cycles. The libraries were purified using Agencourt AMPure XP magnetic beads. The purified RNA-Seq libraries were quantified using the Qubit 1X dsDNA HS Assay Kit. The library size distribution for each library was determined using the Agilent TapeStation D1000 assay (D1000 ScreenTape (5067–5582) with D1000 Reagents (5067–5583). The RNA-Seq libraries were subsequently pooled in equimolar amounts for sequencing. The average fragment size for the pooled library was determined using the Agilent Bioanalyzer HS DNA and the region analysis function of the Bioanalyzer software. The concentration of adaptor-ligated DNA molecules was confirmed by qPCR using the KAPA Library Quantification Kit for Illumina Platforms on the QuantStudio 12K Flex System. A library dilution series of 1:10,000, 1:50,000, 1:250,000, and 1:1250,000 was quantified in triplicate. The size-adjusted library concentration was calculated using the KAPA Library Quantification Kit (Illumina Platforms) Data Analysis Template (v4.14). The RNA-Seq pooled libraries were denatured with 0.2 N NaOH, diluted to 1.5 pM and combined with the denatured PhiX positive control at a spike-in concentration of 1% v/v for the NGS run according to the standard Illumina protocol. The pooled library was loaded on the Illumina NextSeq 550 instrument (serial no. NB552161) and sequenced using a NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles) consisting of the following: Mid Output Flow Cell Cartridge v2.5; Buffer Cartridge v2; Mid Output Reagent Cartridge v2 150 cycles; Accessory Box v2. The sequencer was programmed to perform a paired-end, single-indexed 2 × 76 cycle sequencing run (Illumina Inc., San Diego, CA, USA).

2.6. RNA-Seq Sample Data Analysis

The RNA-Seq read libraries were uploaded to the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) and an automated RNA-Seq Analysis pipeline was used for the QC of the raw reads, trimming, alignment, assembling, and differential expression analysis. A previously published full-genome sequence of *Serratia* sp. HRI was used as a reference genome for the paired-read library input [16]. The Tuxedo pipeline was used based on the tuxedo suite of tools (i.e., Bowtie2, Cufflinks, Cuffdiff) as part of TopHat 2 [17] and the computational analysis of the bacterial RNA-Seq data according to standards set by McClure and co-workers [18]. The differential expression p-values were computed by q-values based on Benjamini–Hochberg correction with a false recovery rate of <1%.

2.7. Uncharacterised MFS-Efflux Pump

Based on the differential expression analysis and screening of the RNA-Seq results, an uncharacterised MFS efflux pump (Detailed in Table 2) was found to be up-regulated at all the time points during the BC treatment. Additionally, only one copy of this gene was found in the genome and so this gene was chosen as a target for further analysis through quantitative real-time expression studies. All the methodology and results analysis was performed following the MIQE guidelines [19]. The treatment conditions included BC treatment at 20 and 90 min and expanded to include two additional QAC biocides, DDAC and Virukill™, with contact times of 20 min. The characteristics of the target gene were as follows.

Table 2. Target gene details and primer sequences used in this study.

Uncharacterised MFS-Type Transporter	
BV-BRC Identifier	fig 2663241.3.peg.999
RefSeq Identifier	GF111_02590
atgagtgcccttacacgccggcgggcgcgcaaaacctggctggcgactttcgctatcgccctgtccacctttaccgtcgtcacggcagaaatgctgcccgtcgggctgctga ctccgatcgtcagtagacctgaatgcgacgatcgccgcgccggattactgggttccctgcccgcctgtttgcccgcgtgttcgccccgctgggtgctgggtgcccggcgca cggatcgccgcagcctgctgatcgctttctgctgctgatcgccccaacctgctggccgccgccacctccatggcgctgctgttactgcgcgcatcctgctgggctt tgcacggcggtatctgggcatcgaggtgggttggcggaacgtctggtgccgccacttccgtaggattggcgctgtccgtcatcttcagcggcgtagcggcagcctcggtg ttcggcgctgccgctcggggtattcctcggcgaagcgctgggctggcgcatggcggtttctggccgtcgccgtcctcgcgcgctgacctgctcctgctgctgtgcgtgctgcgc cgctgccggtcacgcagacggtagctggcggtttattaccgcgcagcgtgcgaatgccgactgctcctcgggctgctgctgacgtttctgctggtgcgggccacttttaggc ctacacctttgtccgcccgtgctgcagacggctgcggcatcgagggcgctgggtggggccgctgctgttcgctacggcgccgcccgtatcggggaattcatcgccg gccaggccgcggccaacggctacgccgaccctggcgctgatcgccctcgggctggcgctcgccgtcctgctgctgccgctgctgggcccacgcgccgctgagcggcagc gccttctcctgctgtggggcatcgccacggcgcgctttccgtttcgtgatggcatggatgctcaaggcgccccgatcggtcgaggctgcctcttcgctgtatatcgcgct gtttaatctggcgatttctcgcggttactggcgggcgggctggtagtgagcggcgaggctgacgataaacggcggtgctgtccggcatcgctgctgctggcggtggcgatc ctgatgcgaacccgcccacaggcgctgacaacggcgggcgaaggccgattcgcccggggtga	
Designed forward primer sequence	5'-TTCCGTTTCGCTGATGGC-3'
Designed reverse primer sequence	5'-TGAACCGCAGGAAATCGC-3'

2.8. Reverse Transcription of RNA to cDNA

For the validation and further analysis after the RNA-Seq, biological replicates of the same RNA extracts were converted to cDNA using the Applied Biosystems™ High-Capacity RNA-to-cDNA™ Kit as per the manufacturer's instructions (Applied Biosystems, Waltham, MA, USA). Thereafter, the cDNA concentrations were measured using the 1X ssDNA Qubit Assay Kit and normalised to 10 ng/μL for each cDNA sample.

2.9. Quantitative Real-Time PCR Using SYBRTM Green Assay

Quantitative PCR (qPCR) was carried out in technical and biological triplicates for the uncharacterised MFS efflux pump gene under each condition using the Applied Biosystems™ PowerUp™ SYBRTM Green Master Mix on an Applied Biosystems QuantStudio™ 5 Real-Time PCR system according to the manufacturer's instructions. Untreated samples were compared to four conditions: treatment with BC for 20 min (BC20), treatment with BC for 90 min (BC90), treatment with DDAC for 20 min (D), and treatment with Virukill™ for 20 min (V). The standard concentrations were confirmed using the 1X ssDNA Qubit Assay Kit and the gene copy numbers were extrapolated using the Thermofisher DNA Copy Number and Dilution Calculator available at <https://www.thermofisher.com/za/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html>, accessed on 9 July 2024.

In addition to the in silico analysis and verification using Geneious 11.1.5 software, the designed primers were tested for non-specific amplification of the *Serratia* sp. HRI cDNA and optimised based on pooled biological replicates using agarose gel electrophoresis and the Applied Biosystems Quant Studio™ 5 Real-Time PCR system. All the qPCR samples were set up and run as described below, followed by a dissociation (melt) curve. Standard ramp speeds were used with the reporter molecule SYBRTM Green and passive reference dye ROXTM with no quencher.

The results were analysed using QuantStudio Design & Analysis 2 (DA2) software version 2. Based on the QC and analysis results, standard curves were constructed by DA2, together with the Cq and gene copy numbers of unknown samples relative to each standard curve generated during each run.

3. Results

3.1. Screening for Novel QAC-Resistance Genes

3.1.1. Total RNA Concentration, Integrity and Purity Assessment

Total RNA extracts were tested for purity, integrity and concentration as depicted in Figure 1 and Table 3.

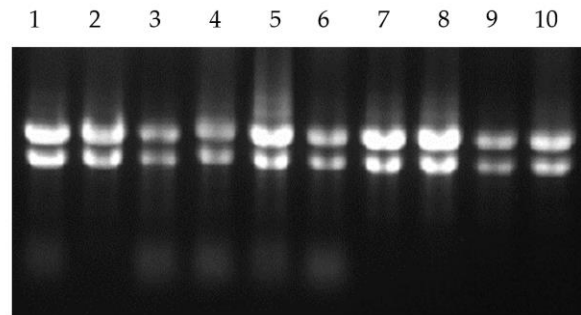


Figure 1. Total RNA extracts visualised on an agarose gel during the RNA extraction optimisation representing the 23s rRNA and 16s rRNA from each sample.

Table 3. RNA samples for the RNA-Seq and qPCR experiments.

Sample	NanoDrop Conc. (ng/μL)	A260/28 0	A260/230	Qubit BR Conc. (ng/μL)	TapeStation RIN
+10	686	2.07	2.27	884	10.0
+20	290	2.19	1.23	398	9.7
+30	400.8	2.18	1.29	552	9.7
+90	638.9	2.15	1.28	876	10.0
D10	571	1.83	1.83	286	8.4
D20	2979	1.51	1.31	1080	9.6
D30	443	1.92	2.25	340	9.1
D90	1076.0	2.19	1.82	1300	10.0

The total RNA extracts passed all QC with high concentrations and low levels of degradation; the purity values were found to indicate high quality for downstream analysis.

3.1.2. Differential Gene Expression (DGE) Analysis

Figures 2 and 3 were generated after the DGE of the samples at different time points. In Figure 2, a great deal of red bars indicate a large number of up-regulated genes, with some down-regulation indicated by green bars. When considering the overall patterns, the highest number of genes differentially expressed, compared to the control, was at 10 min and 30 min of treatment. Figure 3 indicates the changes in gene expression over time in the treated samples and suggests that some genes are differentially expressed over time during treatment; however, not many genes are affected. This indicates that most differential expression is sustained after the 10 min mark.



Figure 2. RNA-Seq differential expression heatmaps compared to the untreated control at four time points. Each line represents a gene: black indicates no differential expression; red indicates an up-regulation; green indicates down-regulation of a gene.

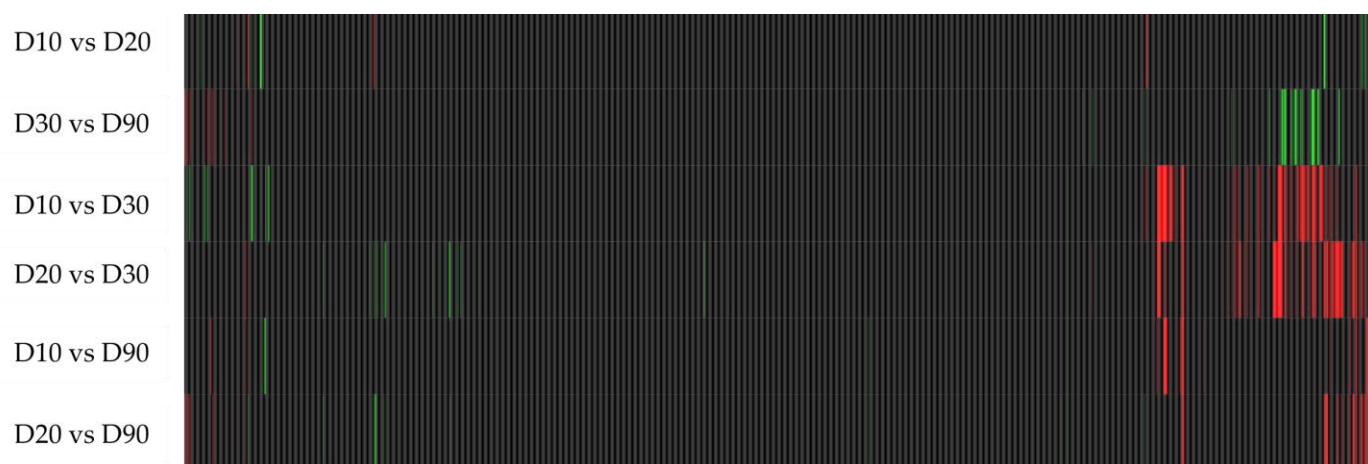


Figure 3. RNA-Seq differential expression heatmaps of the treated samples at different time points. Each line represents a gene: black indicates no differential expression; red indicates an up-regulation; green indicates down-regulation of a gene.

Due to the large numbers of differentially expressed genes, seen in Figure 2 between the control and treated samples, the results were filtered as follows: (1) a fold-change ≥ 3 and Z-score ≥ 3 (a statistical measurement of the differential expression of a gene in relation to the mean of the group). This sub-section of genes, depicted in Figure 3 and quantified in Table 4, were deemed to be the most differentially expressed and were sorted into groups based on the trends of DGE after exposure to BC previously recorded in the literature.

Table 4. Number of genes with a DGE fold-change ≥ 3 and Z-score ≥ 3 compared to the control samples. The genes meeting these criteria were further assigned to four main systems found to be differentially expressed after exposure to BC.

Systems Most Affected in the Literature [21]	10 min	20 min	30 min	90 min
Motility	8	1	4	12
Membrane composition and transport	14	4	11	5
Metabolism adaptations	13	11	11	17
Stress response	7	2	3	3
Uncharacterised	26	18	19	27
Total	68	36	48	64

Table 4 indicates the genes most differentially expressed after exposure to BC at four different time points, where the majority of the genes are uncharacterised for all four time-points.

3.2. qPCR Analysis of Novel QAC-Resistance Target Gene: Uncharacterised MFS Efflux Pump (UMFS)

When searching for target genes for qPCR validation, an uncharacterised MFS efflux pump (RefSeq: WP_017892467.1; gf111_02590) was found to be up-regulated by at least four-fold and to have a Z-score of above 3 at all four time-points. As a result, this gene was chosen as a target for further analysis and validation using qPCR. Samples were collected after exposure to BC at 20 and 90 min as well as DDAC and Virukill™ disinfectants at 20 min of contact time to elucidate more about this gene under different conditions. Template (cDNA) concentration is depicted in Table 5 for all samples after conversion from RNA. Followed by qPCR analysis parameters achieved in Table 6.

Table 5. Sample concentrations after RNA conversion to cDNA for 5 conditions in triplicate.

Treatment	Sample Name	Concentration (ng/μL)
Untreated/Control	+A	93.7
	+B	98.1
	+C	96.8
Benzalkonium chloride (20 min)	B2A	91.9
	B2B	75.4
	B2C	66.5
Benzalkonium chloride (90 min)	B9A	50
	B9B	37.3
	B9C	58.2
DDAC (20 min)	DA	146
	DB	95.8
	DC	120
Virukill™ (20 min)	VA	90.8
	VB	110
	VC	52.8
Negative Reverse Transcriptase	-RT	2 (No change)

Table 6. Characteristics of the standard curves generated for the target gene.

	Standard Curve 1	Standard Curve 2	Standard Curve 3
Slope	-3.584	-3.193	-3.246
Efficiency (%)	90%	106%	103%

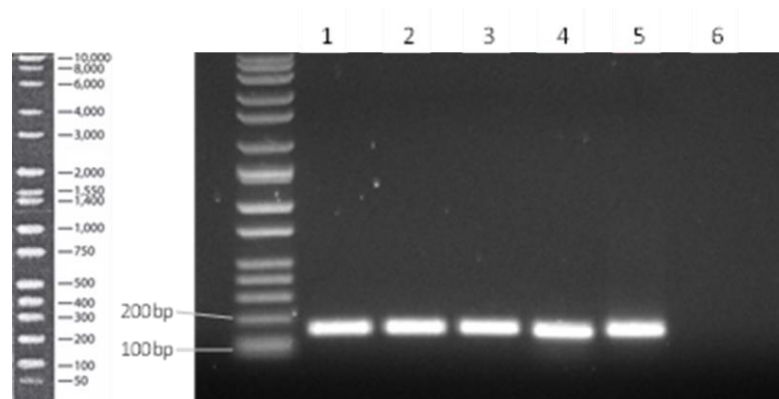


Figure 4. Successful amplification of the uncharacterised MFS efflux pump (UMFS1) target gene from the pooled cDNA biological replicates with the designed primers with no visible non-specific binding. Lane 1: untreated/control pooled sample; Lane 2: BC 20 pooled sample, Lane 3: BC90 pooled sample; Lane 4: DDAC pooled sample; Lane 5: VirukillTM pooled sample; Lane 6: negative reverse transcriptase control.

The results of the real-time PCR analysis depicted in Figure 5 reveal further information about the UMFS1 target gene. An up-regulation of this gene after exposure to BC at the 20 and 90 min time points was confirmed (Table 7), together with an up-regulation in response to the DDAC and VirukillTM disinfectants (Figure 5). Interestingly, the UMFS1 gene was up-regulated at a much higher level at 20 min compared to 90 min.

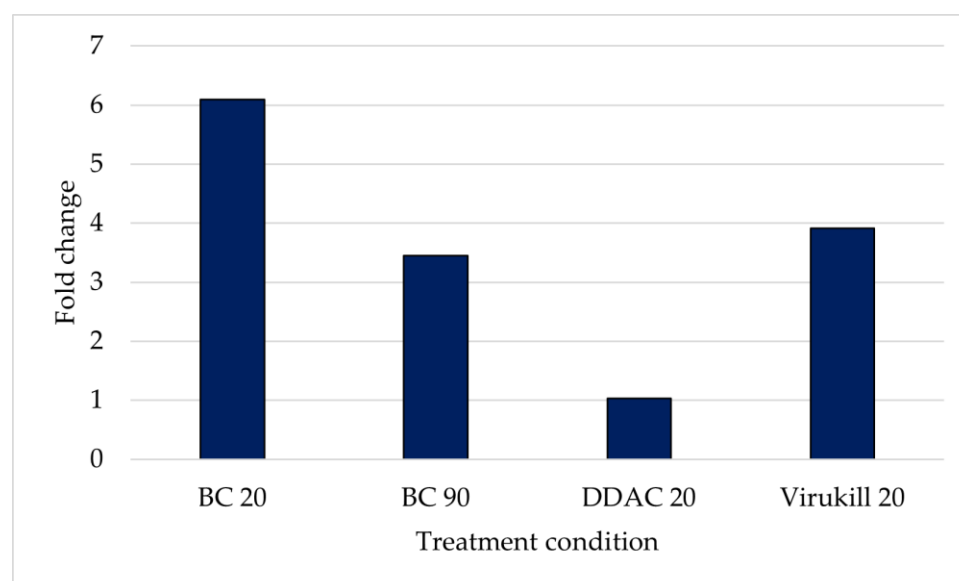


Figure 5. Calculated fold change in the gene expression of UMFS1 gene after exposure to three different QAC-based disinfectants (BC: benzalkonium chloride; DDAC: didecyltrimethylammonium chloride; Virukill: QAC-based disinfectant), at either 20 or 90 min of contact time, compared to untreated samples evaluated by quantitative real-time PCR.

Table 7. Comparison of the calculated fold change in the gene expression of the UMFS1 gene after BC treatments for the RNA-Seq and qPCR data.

Timepoint (min)	Fold Change in Expression RNA-Seq Data	Fold Change Expression qPCR Data
10	5.13279	N/A
20	5.44252	6.09
30	4.16596	N/A
90	4.26023	3.45

4. Discussion

Serratia sp. HRI is an isolate with high resistance capabilities for a wide range of antimicrobials [16]. As such, any insight into the molecular mechanisms of the QAC resistance of this isolate are valuable in the fight against antimicrobial resistance.

Data gathered from RNA-Seq of the transcriptomic response of *Serratia* sp. HRI to benzalkonium chloride shows that the differential gene expression changes over time. The main systems affected were the motility, membrane composition and transport, metabolism adaption, and stress response. The immediate (≤ 10 min) response was characterised by the up-regulation of genes associated with mobile genetic elements, efflux pumps and membrane transporters, biofilm maintenance, pentose phosphate pathway (PPP) metabolites and enzymes, metabolites of the tricarboxylic acid (TCA) cycle and detoxification, antibiotic and resistance genes. After 20 min of exposure, less genes and systems were differentially expressed compared to the initial 10 min readings. The differential expression was observed as a down-regulation of lipoproteins, up-regulation of oxidative and acidic stress responses, and up-regulation of pyruvate production and citrate fermentation feeding into the TCA cycle. After 30 min of exposure, the differential expression profile shifted and more genes were activated or up-regulated, specifically mobile genetic elemental genes, efflux pumps and membrane proteins, copper resistance, biofilm maintenance, TCA intermediates, PPP intermediates, antibiotic and copper resistance genes, and finally, the osmotic stress response. When compared to the reviewed literature, these trends align with an up-regulation of the stress response, efflux pumps and membrane transport, a metabolism shift due to oxidative damage and proteostasis [20]; however, in contrast to the literature, no changes in the expression of iron homeostasis genes were observed for *Serratia* sp. HRI in the short-term (up to 30 min) response to benzalkonium chloride. The long-term (characterised here as 90 min exposure) differential expression patterns in *Serratia* sp. HRI were an up-regulation of genes involved in membrane maintenance, TCA cycle metabolites, NADPH formation, copper and antibiotic resistance, together with the differential expression of mobile genetic elements. Interestingly, all the multidrug and resistance efflux pumps were most differentially expressed within the first 10 min of exposure, suggesting that efflux pumps are an immediate response to biocide treatment but may not be maintained in the long term. This could be due to the high energy and co-substrate demands for the use of efflux pumps.

The aim of the RNA-Seq work was to screen for novel QAC-resistance genes. Tables S1–S4 present a list of novel gene targets, as these genes had the highest observable fold change and greatest Z score, and thus, were most likely to play a role in the high levels of resistance of *Serratia* sp. HRI. A large proportion of the most significantly differentially expressed genes are uncharacterised or classified as hypothetical currently, suggesting a large proportion of the genes involved in QAC resistance remain uncharacterised.

Additionally, many mobile genetic elements or mobile-associated genes were found to be significantly differentially expressed, confirming the high dissemination potential of QAC resistance and the notion that *Serratia* species have an unusually high genome plasticity [22].

Previous work has highlighted that the greater the fold change in the differential expression in RNA-Seq results, the greater the reliability of the result [22,23]. Everaert and co-workers [23] compared the expression of genes evaluated with both RNA-Seq and qPCR and found that the differential expression values of only 1.8% of genes significantly differed in a specific sub-set of genes. The 1.8% of non-comparable genes were all found to be smaller, with fewer exons (in eukaryotic studies), and differentially expressed with less than a two-fold change [22,23]. In light of this, only the genes with the greatest fold changes and highest Z scores were considered, as these would be the most reliable results.

The uncharacterised MFS efflux pump gene introduced in this study was found to be up-regulated at all four timepoints of BC exposure in the RNA-Seq data. Furthermore, the real-time PCR data revealed that this gene was up-regulated for all three QAC biocides used in this study (benzalkonium chloride, DDAC and Virukill™). Interestingly, different levels of up-regulation were noted among the three generations of QAC biocides, indicating slight changes in the expression levels in response to different biocides in the same group. This corresponds to work performed in *Escherichia coli*, where the gene expression profiles differed depending on the type of biocide used [21]. Therefore, this suggests that not only can gene expression differ between groups of biocides but expression profiles may differ between generations of biocides within the same group.

5. Conclusions

The up-regulation of the uncharacterised MFS efflux pump elucidated in this work, as confirmed by RNA-Seq and real-time PCR expression studies, suggests this to be a strong candidate for a novel QAC-resistance gene. Further analysis is recommended to confirm the effect of this efflux pump on the resistance phenotype and the extent of its role in antimicrobial susceptibility to not only QAC-biocides but antibiotics and other antimicrobials as well. This work showcases the power of “omics” tools for future work in screening and identifying novel antimicrobial resistance genes. The molecular characterisation of antimicrobial resistance is vital to the understanding of the epidemiology of resistance. Once resistance genes have been characterised, this information can be used in the surveillance of antimicrobial resistance and in the tracking of the mobility and transfer of resistance determinants, and it can elucidate how resistant populations develop.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microorganisms12091891/s1>, Table S1: Greatest differentially expressed genes of untreated and BC treated samples at 10 min exposure time (Log Fold change > 3 and Z value >3); Table S2: Greatest differentially expressed genes of untreated and BC treated samples at 20 min exposure time (Log Fold change > 3 and Z value > 3); Table S3: Greatest differentially expressed genes of untreated and BC treated samples at 30 min exposure time (Log Fold change > 3 and Z value > 3); Table S4: Greatest differentially expressed genes of untreated and BC treated samples at 90 min exposure time (Log Fold change > 3 and Z value > 3). Figures S1–S3 detail real-time PCR amplification plots, standard curve plots, and melt curve plots. Table S5 depicts qPCR raw data. Figure S1: Primer optimisation for the uncharacterised MFS efflux pump by gradient PCR at four different temperatures, 56 °C, 58 °C, 60 °C and 62 °C; Figure S2: Standard curves generated after real-time PCR amplification of the UMFS1 gene including standards and samples for three biological replicates (a), (b), (c); Figure S3: Dissociation (melt) curves generated after real-time PCR amplification of the UMFS1 gene for all standards, samples and negative

controls; Table S5: Expression and gene count data for all samples. Where no amplification signal was detected, the software output stated “undetermined”.

Author Contributions: Conceptualisation, S.M. and R.B.; methodology, S.M. and C.B.-v.J.; software, S.M.; validation, S.M. and C.B.-v.J.; formal analysis, S.M.; investigation, S.M.; resources, C.B.-v.J. and R.B.; data curation, S.M.; writing—original draft preparation, S.M.; writing—review and editing, C.B.-v.J. and R.B.; visualisation, S.M.; supervision, C.B.-v.J. and R.B.; project administration, R.B.; funding acquisition, C.B.-v.J. and R.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The sequence data are available on request. The RNA-Seq data analysis methodology is freely and openly available at the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) available at “<https://www.bv-brc.org/> (accessed on 10 June 2023).”

Acknowledgments: The authors would like to acknowledge the Department of Genetics at the University of the Free State, South Africa, for the use of their equipment.

Conflicts of Interest: The authors declare no conflicts of interest.

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4.2. Chapter 4 Conclusions

Through sequencing and bioinformatics, a timeline of gene expression could be generated for the response of *Serratia* sp. HRI to benzalkonium chloride. DGE profiles could be tracked over time and characterised into short-term and long-term bacterial responses. This work also generated lists of potential novel QAC resistance genes and their activity at different time points for future study. Compared to literature, the DGE profile for *Serratia* sp. HRI is similar to work done in *Escherichia coli*, where a short-term (20-minute) exposure to BC resulted in an up-regulation in genes associated with proteostasis and multidrug efflux (Merchel Piovesan Pereira et al., 2020). In *E. coli*, DGE was observed for genes involved in motility, transport and amino acid synthesis, aligning with DGE patterns for *Serratia* sp. HRI (Merchel Piovesan Pereira et al., 2020). A long-term (90-minute) exposure of *E. coli* to BC resulted in a down-regulation in motility, similar to *Serratia* sp. HRI (Merchel Piovesan Pereira et al., 2020). The stress response was also found to be differentially expressed in both *Serratia* sp. HRI and *E. coli* after exposure to BC (Merchel Piovesan Pereira et al., 2020). However, there was also a down-regulation of sugar metabolism in *E. coli* (Merchel Piovesan Pereira et al., 2020), in contrast to what was seen for *Serratia* sp. HRI, where sugar uptake and metabolism were up-regulated. The up-regulation of sugar uptake and metabolism could occur to increase ATP levels to support active efflux and other survival mechanisms. In both *Serratia* sp. HRI and *E. coli*, no DGE of biofilm-associated genes were observed at any time. These results support the hypothesis that biofilm formation is not a resistance mechanism put forward in Chapters 1 and 3 of this dissertation.

Many hypothetical, putative and uncharacterised proteins which were significantly differentially expressed in *Serratia* sp. HRI were identified. The uncharacterised proteins identified in this work are attractive targets for future work to identify novel resistance genes. From this list, an uncharacterised MFS efflux pump was confirmed as significantly differentially expressed using two different gene expression analysis platforms. This UMFS gene was up-regulated after exposure to all three QAC disinfectants and interestingly, had a higher level of expression at 20 minutes, which tapered to a lower level of expression at 90 minutes. These findings suggest that efflux pumps in

the MFS family may be an initial response to antimicrobial attack that decreases over time, perhaps stalling until more long-term resistance mechanisms are activated and established. Similar results were observed in *E. coli* when exposed to BC; multidrug efflux and drug transport were among the most significantly up-regulated systems at 20 minutes (Merchel Piovesan Pereira et al., 2020). However, these systems were not identified as those with significant up-regulation at 90 minutes of exposure (Merchel Piovesan Pereira et al., 2020).

The work presented in this chapter strengthens the hypothesis from previous chapters and lays the foundation for future work in identifying novel QAC resistance genes.

4.3. References

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Chapter 5: Transcriptomic analysis of *Serratia* sp. HRI exposed to sub-MIC levels of DDAC & Virukill by RNA-Seq

5.1. Introduction

Transcriptomic analysis in the previous chapter revealed many potential novel BC resistance genes and trends in gene expression over time. This chapter aims to take this work further by continuing with RNA-Seq for *Serratia* sp. HRI after exposure to two additional QAC disinfectants, Didecyltrimethylammonium chloride (DDAC) and Viurkill®. DDAC is a fifth-generation QAC, and Viurkill® is a finished product containing a novel modified version of DDAC (Bragg *et al.* 2014). These disinfectants represent the newer generation QACs; notably, the finished product represents the real-world products used in industry and medical environments today.

This chapter will focus on a broader scope of analysis and, instead of identifying individual resistance genes, will study overall trends after QAC exposure. Interestingly, the results thus far on QAC resistance elucidated in this work seem to link directly or indirectly to the bacterial stress response constantly. In Chapter 1, differential expression profiles of multiple bacteria exposed to BC consistently included the bacterial stress response (McCarlie *et al.* 2023a); the hormetic effect observed in Chapter 2 is hypothesised to be due to the overstimulation of the stress response (McCarlie *et al.* 2023b); in chapter 3 the increased levels of cell nodules were hypothesised to be outer membrane vesicles whose production is increased by the stress response (Macdonald and Kuehn 2013). The DGE profile generated in Chapter 4 found the stress response to be differentially expressed after exposure to BC (McCarlie, Boucher-van Jaarsveld and Bragg 2024). This raises the question: how much of an impact does the stress response really have on QAC susceptibility and resistance? Currently, little to no literature is available on the stress response and its effect on disinfectant susceptibility and resistance.

The bacterial stress response is understood to be a general widespread response to adverse conditions, including cold, heat, acidic conditions, alkaline conditions, osmotic stress, membrane stress and

nutritional stress (Poole 2012; Dam, Pagès and Masi 2018; da Cruz Nizer *et al.* 2021). The stress response impacts gene expression for a multitude of systems and has cell-wide effects, acting as a protective response and influencing metabolism (Darwin 2005; Chan, Boor and Wiedmann 2007; Dam, Pagès and Masi 2018; Davis and Brown 2020). All research on the stress response and its impact on susceptibility has focused on antibiotics up until this point (Lee *et al.* 2009; Nguyen *et al.* 2011; Guest and Raivio 2016; Strugeon *et al.* 2016; Ng *et al.* 2020; Reyes-Fernández and Schuldiner 2020; Mohanraj and Mandal 2022), although the research methodology can be applied in a similar format to study susceptibility to disinfectants. A recent publication by Wiktorczyk-Kapischke and co-workers (2023) revealed the influence of several stressors on reduced susceptibility of *Listeria monocytogenes* to penicillin, meropenem and co-trimoxazole antibiotics. The work presented in this chapter has used a similar research methodology adapted to *Serratia* sp. HRI and *Serratia marcescens* ATCC 13880 to examine the influence of the stress response on QAC susceptibility and resistance.

The experimental findings of this chapter have been published and is entitled “Impact of the Stress Response on Quaternary Ammonium Compound Disinfectant Susceptibility in *Serratia* Species” in the journal *Microorganisms*. This is a peer-reviewed, accredited scientific journal. As the first author, I researched the topic, designed the methodology, conducted the experiments, wrote, and compiled the article. The co-author of this article is my main promoter, Prof. R. R. Bragg, who was integral to the initial conceptualisation as well as review and editing of the paper. This published original article is presented as the complete fifth chapter of this dissertation.



Impact of the Stress Response on Quaternary Ammonium Compound Disinfectant Susceptibility in *Serratia* Species

Samantha McCarlie and Robert R. Bragg *



Abstract: The well-known problem of antibiotic resistance foreshadows a similar threat posed by microbial resistance to biocides such as disinfectants and antiseptics. These products are vital for infection control, yet their overuse during the COVID-19 pandemic has accelerated the development of resistant microorganisms. This study investigates the molecular mechanisms underlying disinfectant resistance in *Serratia* sp. HRI. The transcriptomic responses of *Serratia* sp. HRI were used to identify significant gene expression changes during exposure to QACs and revealed increased methionine transport and polyamine synthesis. Polyamines, crucial in cellular stress responses, were notably upregulated, suggesting a pivotal role of the stress response in disinfectant resistance. Further, our susceptibility tests revealed a marked decrease in susceptibility to QACs under various stress conditions, supporting the hypothesis that stress responses, mediated by polyamines, decrease susceptibility to QACs. This research highlights polyamines as key players in disinfectant resistance, offering novel insights into resistance mechanisms and antimicrobial susceptibility. Our findings emphasise the need for continued investigation into disinfectant resistance and the role of stress responses, particularly polyamine-mediated mechanisms, to direct strategies for preserving disinfectant efficacy and developing future antimicrobial agents.

Keywords: antimicrobial resistance; quaternary ammonium compounds; polyamines; RNA-Seq



Citation: McCarlie, S.; Bragg, R.R. Impact of the Stress Response on Quaternary Ammonium Compound Disinfectant Susceptibility in *Serratia* Species. *Microorganisms* **2024**, *12*, 2240.

<https://doi.org/10.3390/microorganisms12112240>

Academic Editor:
Akikazu Sakudo

Received: 19
August 2024
Revised: 31

1. Introduction

Disinfectants are crucial for infection control and preventing food-borne illnesses in healthcare, agriculture, and the food industries. The COVID-19 pandemic highlighted our reliance on biocides, such as disinfectants and antiseptics, as part of biosecurity and infection control. During the pandemic, the use of disinfectants increased drastically, creating greater selective pressure for resistance development [1,2]. Additionally, the use of poor-quality disinfectants during the pandemic has led to a rise in the rates of disinfectant-resistant microorganisms [3,4]. Currently, the most significant impact of resistance to disinfectants can be seen in the rapidly rising rates of hospital-acquired infections around the globe due to impaired infection control procedures and products [3]. *Serratia marcescens* has emerged as an opportunistic nosocomial pathogen causing various types of infections [5] and has gained a notorious reputation for causing hospital-acquired infections in paediatric and neonatal wards [6–10]. This opportunistic pathogen has been isolated on several occasions from bottles of disinfectants used in hospitals, revealing its ability to survive within bottles of disinfectants [9,11–13]. Its ability to survive and cause infection within the healthcare setting has been attributed to intrinsic and acquired antimicrobial resistance within this genus [5,14–16]. Current research into antimicrobial resistance has focused on antibiotics, with resistance to disinfectants as a relatively novel field of study that has gained more attention post-COVID-19. In light of this, our understanding of the molecular mechanisms of disinfectant resistance is still poor, and current hypotheses require more evidence.

Once the mechanisms of the resistance phenotype are understood, this knowledge can be used to safeguard current disinfectants and direct the design of future antimicrobial compounds.

Quaternary ammonium compounds (QACs) were the largest group of disinfectants approved for use during the COVID-19 pandemic by the EPA [17]. They function by targeting the cell membrane of microorganisms and result in the formation of reactive oxygen species (ROS), which cause further cellular damage [18,19]. QACs remain the focus of most studies on resistance to disinfectants, and slowly, the molecular mechanisms are being uncovered [20–23]. Gene expression studies are gaining traction in the study of antimicrobial resistance as RNA-Seq techniques advance, becoming more accurate and reliable, and further validation is only required at very low levels of differential expression [24]. This robust technique reveals which systems and individual genes are most differentially expressed upon exposure to antimicrobials and uncovers insights into resistance mechanisms.

In industries dependent on infection and quality control, physical agents are used to hinder microbial growth, such as high or low temperatures, desiccation, and osmotic pressure [25,26]. Chemical agents include disinfectants and control microbial growth through widespread membrane and oxidative damage [18,19]. These adverse conditions activate the bacterial stress response, which can affect susceptibility to antibiotics [27–32]. Recent work highlighted the stress response as a key system differentially expressed after exposure to QAC disinfectants [33]. However, little to no work has been completed to evaluate the effect of stressors on disinfectant susceptibility and resistance.

In this study, disinfectant-resistant isolate *Serratia* sp. HRI [34] was used to elucidate the molecular mechanisms of QAC resistance through transcriptomics. Thereafter, the impact of the stress response on the susceptibility to a QAC product was evaluated together with the most closely related type strain, *Serratia marcescens* ATCC 13880.

2. Materials and Methods

Serratia sp. HRI exhibits high QAC resistance and thus provides a unique opportunity to study bacterial resistance to QAC-based disinfectants [34]. As a control, the most closely related type strain, *Serratia marcescens* ATCC 13880, is used as a susceptible strain for comparison [34].

2.1. Microorganisms

The bacteria used in this study are highly resistant isolate *Serratia* sp. HRI [34] from the University of the Free State culture collection and the most closely related type strain, *Serratia marcescens* subsp. *marcescens* ATCC 13880, obtained from the American Type Culture Collection. *Serratia* sp. HRI was selected for study due to high levels of QAC resistance, providing an opportunity to elucidate QAC-resistance mechanisms [34].

2.2. Disinfectants

For this study, two disinfectants were used: Virukill™ (Didecyldimethylammonium Chloride 120 g/L) and DDAC (800 g/L Didecyldimethylammonium Chloride) were obtained from ICA International Chemicals, Stellenbosch, South Africa. The active ingredient DDAC was used to evaluate QAC susceptibility for only the active ingredient (pure QAC), whereas Virukill® is a registered product, with additional additives, and therefore, represents a commonly used QAC-based infection control product.

2.3. Bacterial Cultivation for RNA-Seq

A pre-inoculum of *Serratia* sp. HRI was prepared by transferring a single colony from 24 h agar plates to 25 mL Luria Broth (LB) medium (10 g/L Merck Tryptone, 10 g/L Merck sodium chloride, 5 g/L Merck yeast extract, Darmstadt, Germany) in 250 mL Erlenmeyer flasks and incubated at 37 °C for 16 h. From this pre-inoculum, 2.5 mL was added into 500 mL Erlenmeyer flasks containing 47.5 mL LB media and incubated at 250 r min⁻¹ and 37 °C for 60 min to ensure cultures would enter an exponential phase of growth based on previous studies [35]. After 60 min, Minimal Inhibitory Concentration (MIC) levels of DDAC (76.62 mg/L, South Africa) or VirukillTM (1200 mg/L, South Africa), determined previously [34], were added to the test cultures, and a contact time of 20 min was allowed before the cell pellets were immediately treated with Invitrogen RNAlater Stabilization Solution (Carlsbad, CA, USA) to store and stabilise RNA within the cells before RNA extraction commenced.

2.4. RNA Extraction and Quality Control

RNA extraction was completed on fresh cell pellets using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions in biological triplicates for untreated (control) and disinfectant-treated samples. An additional on-column DNase digestion was performed to eliminate possible genomic DNA (gDNA) contamination using the ZymoResearch (Irvine, CA, USA) DNase I set per the manufacturer's instructions. RNA quantity and quality were evaluated on agarose gels, a NanoDrop 1000 spectrophotometer and the Qubit RNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Once all samples had passed QC based on RNA integrity, concentration, and purity, the samples were sent to the Centre for Proteomic and Genomic Research for sequencing (Cape Town, South Africa) and were prepared as described below.

Before ribosomal depletion, the total RNA for all samples was precipitated by adding sodium acetate (0.3 M) and 3 volumes of ice-cold absolute ethanol. The RNA was allowed to precipitate at -80 °C overnight. RNA was pelleted by centrifugation at 13,000× g for 10 min. RNA pellets were washed twice with ice-cold 70% ethanol and centrifuged at 13,000× g for 10 min. Ethanol was removed, and the pellets were dried at room temperature. The pellets were then resuspended in nuclease-free water. RNA was re-evaluated for purity using the NanoDrop 8000 spectrophotometer, quantity using the Qubit RNA BR Assay Kit (Waltham, MA, USA), and integrity using the Agilent Bioanalyzer RNA 6000 Nano Kit (Waldbronn, Germany).

Total RNA extracts underwent Ribosomal depletion, RNA-Seq library preparation and QC, RNA-Seq library pooling, and RNA-Seq library sequencing as elaborated in Appendix A.

2.5. RNA-Seq Data Analysis

RNA-seq data were obtained from bacterial species following sequencing on Illumina (San Diego, CA, USA). The raw sequencing reads were quality-checked using FastQC. The adapter sequences were removed using Trimmomatic as part of the Trinity pipeline. Trinity (version 2.15.1) software was used for de novo transcriptome assembly with the default parameters. The BUSCO software (available at <https://busco.ezlab.org/>) was executed to assess the completeness of the de novo assembly using a reference bacterial dataset from OrthoDB (bacteria_odb10). Upon completion of the BUSCO analysis, the output directory contains reports and metrics detailing the completeness of the transcriptome assembly. Key metrics such as the percentage of complete single-copy and duplicated BUSCOs, fragmented BUSCOs, and missing BUSCOs were evaluated to assess the quality and completeness of

the assembly. The UniProt database (2024_02 release) of reviewed proteins (Swiss-Prot) was used as the reference database for alignment using diamond (v2.1.9) software. Transdecoder (v5.7.1) was used to predict ORFs and the protein-coding sections of the identified transcripts in the Trinity transcriptome. The predicted protein-coding frames from TransDecoder were used as input to align to the UniProt database.

The newly assembled transcriptome, in FASTA format, was indexed using Bowtie2-build to create a Bowtie2 index. The indexed transcriptome was used as the reference for aligning RNA-seq reads using Bowtie2. Paired-end reads from the RNA-seq data were aligned to the transcriptome. The RSEM software (version 1.3.1) was employed to estimate transcript abundances based on the aligned RNA-seq reads. The edgeR package was used to determine the p-value and False Discovery Rate (FDR) for the given transcripts for all the different conditions and positive control samples. Lowly expressed transcripts were removed, and the TMM normalisation technique was employed as part of the edgeR package. Since the samples were technical replicates, a constant dispersion estimate of 0.01 was used during the DGE analysis. A volcano plot was used for visualisation. The graphs were generated by using R and custom scripts. This approach applied thresholds to DEG, including log fold change ($\log_{2}FC \geq 1$ or ≤ -1) (corresponding to 2-fold upregulation or downregulation, respectively), $p\text{-value} < 0.05$, and $FDR < 0.05$.

2.6. Minimal Inhibitory Concentration Susceptibility Tests

Minimal inhibitory concentrations (MICs) for Virukill™ (South Africa) were determined before and after stress treatments for *Serratia* sp. HRI and *Serratia marcescens* ATCC 13,880. MICs were performed by the broth dilution method [36], which started with a fresh overnight culture that had been standardised to the McFarland standard of 0.5. The disinfectants were serially diluted 2-fold with sterile water in a 96-well microtitre plate, and a 10% inoculum of the bacterium was added to the disinfectant dilutions. The 96-well plates were incubated at 37 °C for a 20 min contact time. Thereafter, a 10% inoculum was transferred into a new 96-well microtitre plate containing BHI growth media and incubated at 37 °C overnight. The MIC was determined to be the lowest concentration at which no visible bacterial growth occurred. The average MIC values were calculated from at least 4 technical repeats and a minimum of 3 biological replicates for each bacterium against each disinfectant.

2.7. Selected Stress Factor Procedures

All bacterial cultures were prepared overnight in a pre-inoculum in BHI growth media before being subjected to stress treatments in biological triplicates. The bacterial cultures were subject to one of seven different stress treatments and standardised to the McFarland standard before the post-treatment MICs were completed. Osmotic stress was induced by the addition of 3.5% NaCl to growth media for 24 h. Cold stress was induced by storing cultures at 4 °C for 48 h. Acidic conditions were created by adjusting the pH of the growth media to pH 5.5 for 30 h. Alkaline conditions were brought about by adjusting the pH of the growth media to pH 8.5 for 30 h. Heat stress was induced by incubating cultures at 55 °C for 20 min. Envelope stress was induced by the addition of 9.8 mg/mL benzalkonium chloride and 0.098 mg/mL benzalkonium chloride to *Serratia* sp. HRI and *Serratia marcescens* ATCC, respectively, for 40 min. Lastly, oxidative stress was induced by adding 0.1 mM H₂O₂ for 20 min.

3. Results

3.1. RNA-Seq Differential Expression Analysis

3.1.1. *Serratia* sp. HRI Treated with DDAC Disinfectant Compared to Untreated Controls

Figure 1 and Table 1 depict the DGE patterns and results between the table.

AC-treated bacteria and untreated bacterial samples. Differential gene expression patterns in Figure 1 depict a large number of genes differentially expressed after treatment with the majority of differential expression being upregulation. The most significantly differentially expressed genes were labelled in Figure 1, and details are presented in Table 1. The gene functions annotated in Table 1 revealed that treatment with the DDAC disinfectant led to an increase in the expression of ABC transporters associated with methionine import, along with an increased expression of genes associated with protein and polyamine synthesis, as well as increased options for substrate use as a carbon source.

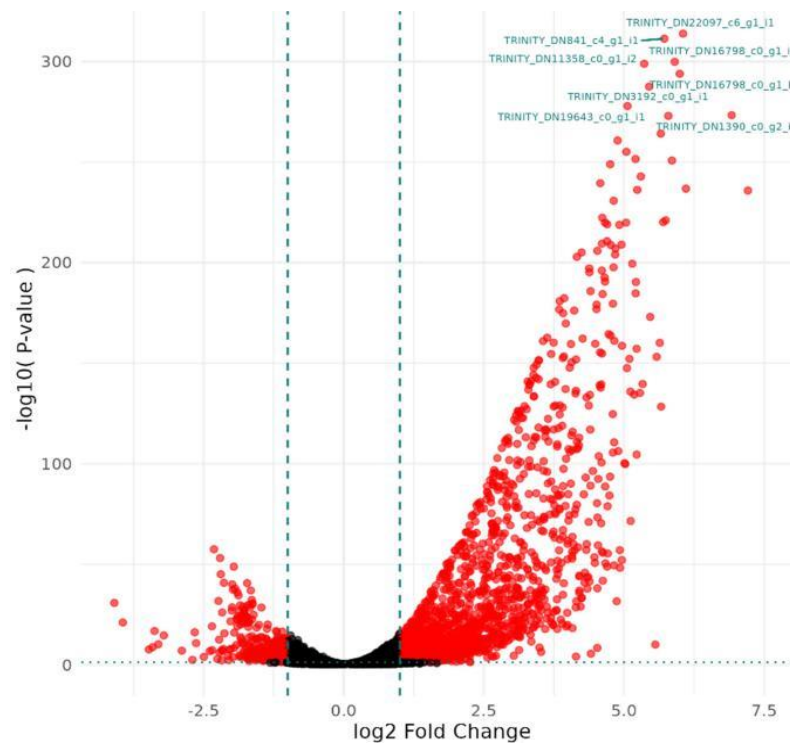


Figure 1. A volcano plot of differentially expressed genes in the DDAC-treated samples plotted by fold change and statistical significance compared to the untreated samples.

Table 1. Transcripts with the greatest differential expression (as identified in Figure 1) in DDAC-treated samples compared to untreated control samples annotated by using the UniProt database.

Protein Name	Function
Methionine import ATP-binding protein MetN 2 (EC 7.4.2.11)	A component of the ABC transporter complex MetNIQ involved in methionine import, functions in energy coupling to the transport system. {ECO:0000255 HAMAP-Rule:MF_01719}.
Dihydroxy-acid dehydratase (DAD) (EC 4.2.1.9)	Involved in the biosynthesis of branched-chain amino acids by catalysing the dehydration of (2R,3R)-2,3-dihydroxy-3-methylpentanoate (2,3-dihydroxy-3-methylvalerate) into 2-oxo-3-methylpentanoate (2-oxo-3-methylvalerate) and of (2R)-2,3-dihydroxy-3-methylbutanoate (2,3-dihydroxyisovalerate) into 2-oxo-3-methylbutanoate (2-oxoisovalerate), the penultimate precursor to L-isoleucine and L-valine, respectively. {ECO:0000255 HAMAP-Rule:MF_00012}.
Inducible ornithine decarboxylase (EC 4.1.1.17)	The first enzyme leading to putrescine and thus polyamine synthesis. {ECO:0000305}.
Glycerol dehydrogenase (GDH) (GLDH) (EC 1.1.1.6)	Catalyses the NAD-dependent oxidation of glycerol to dihydroxyacetone (glycerone, allowing for the utilisation of glycerol as a carbon source. Additionally, responsible for the oxidation of 1,2-propanediol and 2,3-butanediol and the reduction in dihydroxyacetone. {ECO:0000269 PubMed:7635824}.
Translation initiation factor IF-3	IF-3 binds to the 30S ribosomal subunit and results in a shift of the equilibrium between the 50S and 30S subunits for 70S ribosomes, enhancing the availability of 30S subunits and protein synthesis initiation. {ECO:0000255 HAMAP-Rule:MF_00080}.

3.1.2. *Serratia* sp. HRI Treated with Virukill™ Disinfectant Compared to Untreated Controls

Figure 2 and Table 2 depict the DGE patterns between Virukill™-treated bacteria and untreated bacterial samples. Differential gene expression patterns in Figure 2 depict a large number of genes differentially expressed after treatment with the majority of differential expression being upregulation. The most significantly differentially expressed genes were labelled in Figure 2, and details are presented in Table 2. The gene functions annotated in Table 2 revealed that Virukill®-treated bacteria upregulated genes associated with amino acid and protein synthesis and ABC transporters linked to methionine import.

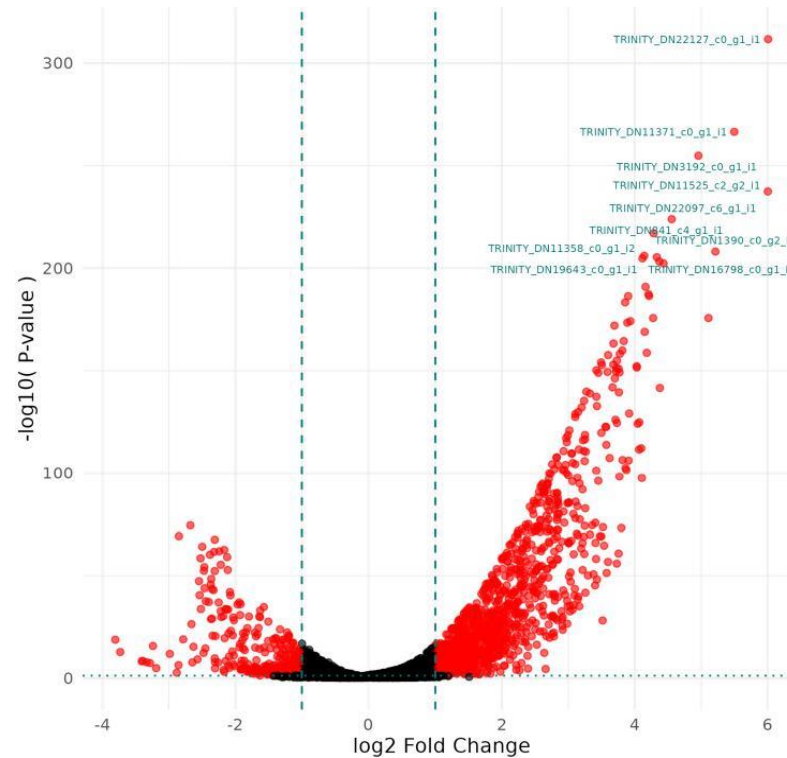


Figure 2. A volcano plot of differentially expressed genes in the Virukill™-treated samples plotted by significance and fold change compared to the untreated samples.

Table 2. Transcripts with the greatest differential expression (labelled in Figure 2) between Virukill™-treated samples and untreated control samples as annotated by the Uniprot database.

Protein Name	Function
Small ribosomal subunit protein bS16 (30S ribosomal protein S16)	
Methionine import ATP-binding protein MetN 2 (EC 7.4.2.11)	A component of the ABC transporter complex MetNIQ involved in methionine import, functions in energy coupling to the transport system. {ECO:0000255 HAMAP-Rule:MF_01719}.
Large ribosomal subunit protein uL15 (50S ribosomal protein L15)	Binds to the 23S rRNA. {ECO:0000255 HAMAP-Rule:MF_01341}.
Protein translocase subunit SecY	The central subunit of the protein translocation channel SecYEG. {ECO:0000255 HAMAP-Rule:MF_01465}.
Large ribosomal subunit protein uL2 (50S ribosomal protein L2)	A critical rRNA binding protein, needed for association of the 30S and 50S subunits to form the 70S ribosome, tRNA binding, and peptide bond formation. It may have peptidyltransferase activity. Forms multiple interactions with the 16S rRNA in the 70S ribosome. {ECO:0000255 HAMAP-Rule:MF_01320}.
Dihydroxy-acid dehydratase (DAD) (EC 4.2.1.9)	Plays a role in the biosynthesis of branched-chain amino acids, catalyses the dehydration

of (2R,3R)-2,3-dihydroxy-3-methylpentanoate (2,3-dihydroxy-3-methylvalerate) into 2-oxo-3-methylpentanoate (2-oxo-3-methylvalerate) and of (2R)-2,3 dihydroxy-3-methylbutanoate (2,3-dihydroxyisovalerate) into 2-oxo-3-methylbutanoate (2-oxoisovalerate).

3.1.3. *Serratia* sp. HRI DDAC Treatment Compared to Virukill™ Treatment

Figure 3 and Table 3 depict the DGE patterns and genes with the most significant differential expression between the DDAC- and Virukill™-treated samples. These genes are most different in the bacterial response to the two disinfectant treatments. Increased expression was seen in genes associated with polyamine synthesis, general protein and carbohydrate transport, and putrescine and ascorbate transport. In addition, an increase in substrate utilisation for a carbon source was seen.

Although Virukill® utilises DDAC as its active compound, as a final product used in industry, it contains many additional compounds in its formulation. Therefore, any changes in susceptibility and DGE profiles can be attributed to the modified formulation and additives in the final disinfectant product.

RNA-Seq data in Tables 1–3 revealed strong differential expression in polyamine synthesis and transport. An analysis of the literature elucidated the links between polyamines and the stress response in reducing susceptibility to antibiotics. However, a lack of research has been completed to reveal whether this is the case for disinfectant antimicrobials as well. Therefore, susceptibility tests were completed to determine what effect the stress response has on disinfectant susceptibility.

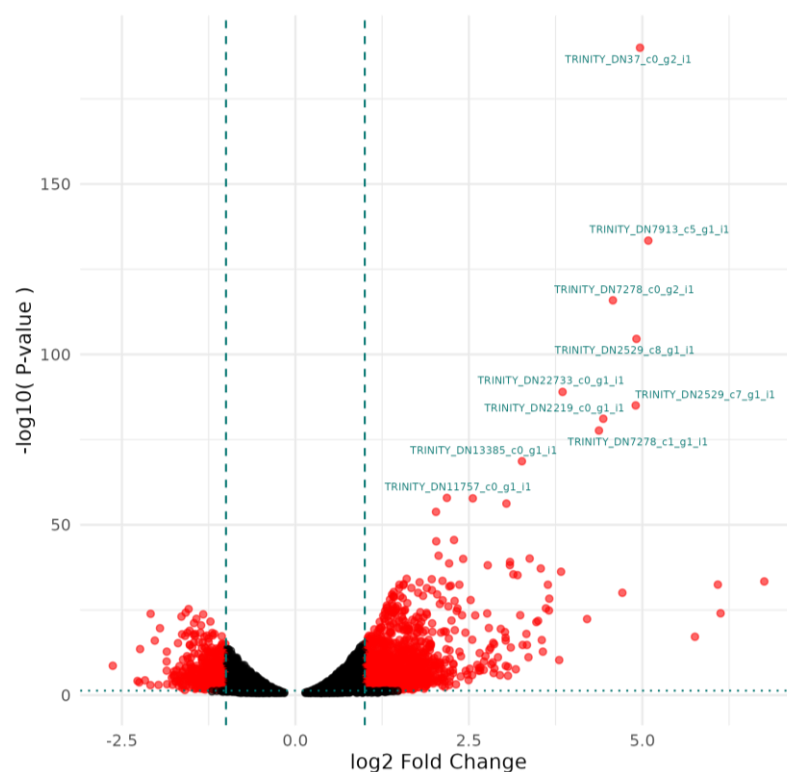


Figure 3. A volcano plot of differentially expressed genes between Virukill™- and DDAC-treated samples plotted by significance and fold change.

Table 3. Transcripts with the greatest differential expression (as labelled in Figure 3) between DDAC-treated and Virukill™-treated samples as annotated by the Uniprot database.

Protein Name	Function
Inducible ornithine decarboxylase (EC 4.1.1.17)	The first enzyme leading to putrescine and thus polyamine synthesis. {ECO:0000305}.
Putrescine transporter PotE (Putrescine-proton symporter/putrescine-ornithine antiporter)	Catalyses both the uptake and excretion of putrescinedependent on the membrane potential and putrescine-ornithine antiporter activity, respectively. {ECO:0000255 HAMAP-Rule:MF_02073}.
Inducible lysine decarboxylase (LDC) (EC 4.1.1.18)	
Dipeptide and tripeptide permease C (Dipeptide/tripeptide: H (+) symporter DtpC)	Proton-dependent permease that transports di- and tripeptides, with significantly higher specificity towards dipeptides. A further preference exists for dipeptides with a C-terminal Lys residue. This protein can bind Ala-Lys, Lys-Ala, Ala-Ala, and transport alanine and trialanine. {ECO:0000269 PubMed:19703419, ECO: 0000269 PubMed: 21933132, ECO: 0000269 PubMed: 22940668, ECO: 0000269 PubMed: 24440353}.
PTS system trehalose-specific EIIBC component (EIIBC-Tre) (EII-Tre) [Includes: Trehalose-specific phosphotransferase enzyme IIB component (EC 2.7.1.201) (PTS system trehalose-specific EIIB component); Trehalose permease IIC component (PTS system trehalose-specific EIIC component)]	The phosphoenolpyruvate-dependent sugar phosphotransferase system (sugar PTS), a major carbohydrate active transport system, catalyses the phosphorylation of incoming sugar substrates concomitantly with their translocation across the cell membrane. This system is involved in trehalose transport at low osmolarity. {ECO:0000269 PubMed:2160944, ECO: 0000305 PubMed:7608078}.
Glycerol dehydrogenase (GDH) (GLDH) (EC 1.1.1.6)	Catalyses the NAD-dependent oxidation of glycerol to dihydroxyacetone (glycerone, allowing for the utilisation of glycerol as a carbon source. Additionally, responsible for the oxidation of 1,2-propanediol and 2,3-butanediol and the reduction in dihydroxyacetone. {ECO:0000269 PubMed:7635824}.
Aspartate ammonia-lyase (Aspartase) (EC 4.3.1.1)	
Ascorbate-specific PTS system EIIC component (Ascorbate-specific permease IIC component UlaA)	Functions as a catalyst for the phosphorylation of incoming sugar substrates simultaneously with their translocation across the cell membrane. Specifically, the enzyme II UlaABC PTS

	system is involved in ascorbate transport. {ECO:0000250 UniProtKB:P39301}.
Nitrate reductase molybdenum cofactor assembly chaperone NarJ (Redox enzyme maturation protein NarJ)	The chaperone required for correct molybdenum cofactor insertion and final assembly of the membrane-bound respiratory nitrate reductase 1. {ECO:0000250}.

3.2. Effect of Stressors on Disinfectant Susceptibility

Serratia sp. HRI (resistant) and *Serratia marcescens* ATCC (type strain) were exposed to seven different stress conditions before and after susceptibility tests were completed to determine if any changes in susceptibility occurred after exposure to a stressor.

Susceptibility tests for the resistant isolate are shown in Figure 4, and *Serratia* sp. HRI showed significant increases (99% confidence) in MICs for the QAC-based disinfectant after all seven stressors.

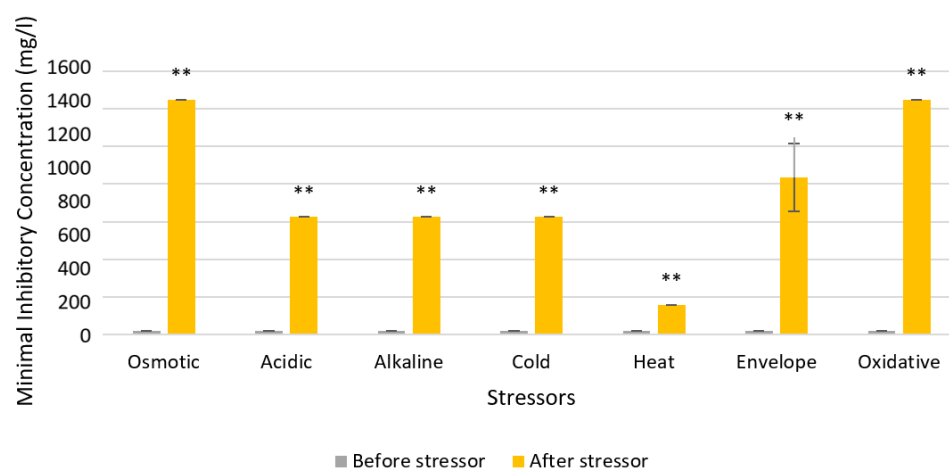


Figure 4. Changes in susceptibility to QAC-based disinfectant Virukill™ for *Serratia* sp. HRI after exposure to one of seven stressors. Statistically significant differences (99% confidence) in MIC after stress condition are labelled with a double asterix.

When assessing the type strain, MICs for the QAC-based product in Figure 5 increased after cold stress, and the remaining six stressors increased the MIC significantly (99% confidence). For both the resistant isolate and type strain, MICs against QAC-based disinfectant Virukill™ increased significantly after exposure to stressors.

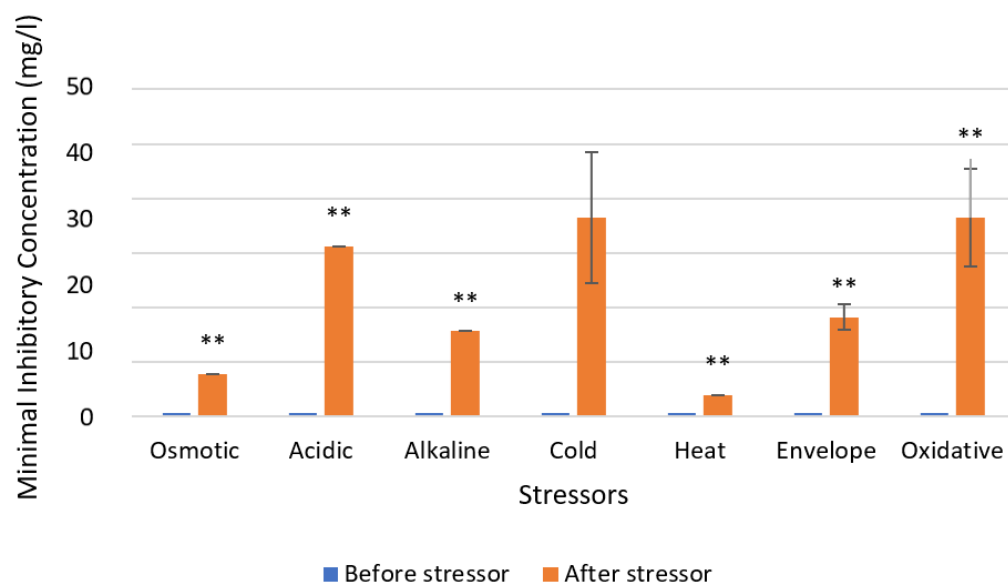


Figure 5. Changes in susceptibility to QAC-based disinfectant Virukill™ for *Serratia marcescens* ATCC after exposure to one of seven stressors. Statistically significant differences (99% confidence) in MIC after stress condition are labelled with a double asterix.

4. Discussion

Analysis of gene expression by RNA-Seq is a powerful tool to uncover novel molecular resistance mechanisms [30]. However, as RNA-Seq focuses on quantifying transcripts, gene expression results may not always translate to a phenotypic response [37,38]. In this work, we have identified possible molecular mechanisms of resistance at a gene expression level and used susceptibility tests to test this hypothesis on a phenotypic level.

The transcriptomic analysis results summarised in Tables 1–3 revealed the genes with the most significant differential expression during treatment with two QAC-based disinfectants, namely, Virukill® and DDAC. Transcripts from the DDAC- and Virukill®-treated samples were characterised by increased methionine transport as part of the ABC transporter complex and increased polyamine synthesis. DDAC-treated samples were further differentiated from Virukill™-treated samples by an additional five-fold increase in polyamine synthesis. Differences in DGE between DDAC and the product Virukill® can be attributed to additives and modifications in the final product. These differences emphasise the importance of research focusing on final products in use instead of isolated active ingredients. A similar upregulation of polyamine transport was observed in *Streptococcus pneumoniae* in response to hydrogen peroxide treatment and physiological temperature stress [39].

During treatment with QAC disinfectants, ROS are produced [18,19], which leads to the oxidation of methionine (Met) residues in proteins, resulting in altered protein structures and impaired protein function [40]. Therefore, the upregulation of methionine transporters could replenish methionine oxidised by ROS to maintain proteostasis. Additionally, there is an integral connection between methionine and polyamine metabolism through S-adenosylmethionine (SAM) [41,42]. Methionine is the precursor for SAM, a crucial substrate for the synthesis of polyamines [43,44]. Polyamines, such as spermidine, spermine, and putrescine, are essential for various cellular functions, including cell growth, division, and the stress response [45]. Polyamine synthesis (upregulated in the transcriptomic data) involves a series of enzymatic reactions starting from SAM [43–45]. Methionine availability, which is impacted by methionine import (upregulated here), influences polyamine synthesis, and polyamine levels can impact methionine metabolism through feedback mechanisms [43,44].

Polyamines are simple primordial stress molecules [43]. They bring about reduced susceptibility to antibiotics by affecting permeability across the cell membrane, thereby modulating antibiotic uptake, binding to antibiotics interfering with the action of fluoroquinolones, influencing bacterial virulence by affecting biofilm formation and toxin production, acting as free radical scavengers, and lastly, influencing genes involved in antibiotic resistance [32,44–47]. However, this effect does differ between bacterial species, the type of antibiotic, and the concentration of polyamines [32]. Polyamines have been linked to susceptibility and resistance to antibiotics. However, little information exists on the effect of polyamines on susceptibility and resistance to disinfectants and other biocides. A recent paper by Short and co-workers [48] described an *Acinetobacter baumannii* disinfectant resistance protein, AmvA, as a spermidine and spermine polyamine efflux pump for the first time. This supports the notion that polyamines, through the stress response, affect susceptibility to disinfectant antimicrobials. However, further research is needed to confirm the link between the stress response and disinfectant susceptibility.

Differences in susceptibility and DGE profiles for DDAC and Viurkill® highlight the need to do research on antimicrobial susceptibility to final products. Research focusing on products that are currently in use in industry allows for the results to be applicable to industry and “real-world” environments. Many antimicrobials are used as part of formulations in final products, and as such, any research that hopes to be relevant to hospital environments or agriculture needs to be completed on the products currently used in these environments. Therefore, further susceptibility tests were completed using the final product and with stressors as would be applicable environmentally.

The stress MICs in Figures 4 and 5 address this research gap, using *Serratia* sp. HRI, a resistant isolate and *Serratia marcescens* ATCC as the most closely related type strain. MIC tests were conducted for a QAC-based Virukill™ disinfectant. The susceptibility of the resistant isolate to the QAC disinfectant decreased significantly for all seven stressors, with each stress condition leading to increased resistance to the QAC disinfectant. In particular, osmotic and oxidative stress conditions resulted in a 60-fold increase in resistance to the disinfectant, as depicted in Figure 4. This trend was also observed in the type strain, with significant decreases in susceptibility to the QAC disinfectant for all stress conditions except cold stress, which decreased susceptibility but did not reach the significance threshold. Thus, these results show that stress conditions decrease susceptibility significantly to the QAC disinfectant for both bacteria and suggest that the stress response is responsible for up to 60-fold reduced susceptibility to the QAC.

The literature suggests that susceptibility linked to the stress response differs between disinfectant active ingredients. In a recent study on uropathogenic *Escherichia coli*, the oxidative stress response was induced after exposure to benzalkonium chloride (QAC), hydrogen peroxide, and triclosan but not chlorhexidine, resulting in different stress responses between biocides [49].

Together, the transcriptomic data and susceptibility tests point to the stress response as a major player in susceptibility and resistance to disinfectants, modulated by polyamines, as observed in the gene expression profiles of Tables 1–3. When considering the impact of polyamines on antimicrobial resistance, the role of polyamines in enhancing antioxidant defence mechanisms and subsequent oxidative stress tolerance in plants and mammals is evident [43]. However, there is a research gap in the impact of polyamines and the stress response on disinfectant susceptibility.

Proposed mechanisms on how polyamines link to reduced susceptibility and resistance to disinfectants and biocides are cell membrane protection, free radical scavenging, and modulation of the stress response. Many disinfectants (such as QACs) function by disrupting the cell membrane. With their positively charged amine groups, polyamines interact with negatively charged phospholipids and stabilise the cell membrane. Additionally, polyamines can interact with membrane proteins, influencing their structure and function, stabilising protein conformation and preventing protein denaturation within the cell membrane. For oxidative disinfectants, such as hydrogen peroxide and hypochlorite, ROS cause cellular damage. Polyamines have antioxidant properties and may mitigate ROS damage by disinfectants, which contributes to reduced susceptibility and resistance. Finally, polyamines are known to have major impacts on the gene expression of key genes in the bacterial stress response, including *rpoS*, which promotes persister cell formation [45], *oxyR*

and *soxRS*, and the subsequent expression of *ahpC*, *katG*, and *katE*, which are integral in resistance to oxidative stress [47,50]. By degrading these molecules, the expression of these genes by polyamines helps maintain cellular integrity and prevent damage to DNA, proteins, and lipids [45].

Links between polyamines and the stress response are evident, as they function as stress modulators [50]. Furthermore, the stress response has been shown to reduce susceptibility to antibiotics [51–53]. This work has taken this notion further, confirming the link between the stress response and susceptibility to QAC-disinfectant compounds. This is supported by the changes in susceptibility profiles after stressors and gene expression analysis indicating polyamines as the mediators of susceptibility changes to disinfectants. Although some work has been completed on polyamine effects and antibiotics [32], this has not been the case for disinfectant resistance and represents a novel molecular mechanism for this group of antimicrobials.

The results obtained from transcriptomic and susceptibility data point to a greater understanding of the disinfectant mode of action and mechanisms of resistance. As the threat of antimicrobial resistance grows, these findings could be used to direct future formulations of disinfectants and other antimicrobials. For example, the development of

efflux pump inhibitors [54–57], which could be used in combination with disinfectants and antimicrobials designed to target the stress response [58].

The results and conclusions of this study are limited to the *Serratia* genus and QAC-based disinfectants during aerobic cultivation. In addition, more stressors could be tested at different contact times and severity to reveal the effect on susceptibility. It is unknown whether similar effects will be seen in other bacteria or with different disinfectant compounds, as the literature on this topic is severely limited. However, future work is highly encouraged to further elucidate the effect of various stressors on disinfectant susceptibility.

5. Conclusions

In this study, the most differentially expressed genes for QAC-based Virukill®- and DDAC-treated cells were identified in QAC-resistant isolate *Serratia* sp. HRI. The differential gene expression profile uncovered strong links to the stress response in the form of polyamine and methionine synthesis and transport in the bacterial response to QAC treatment. Stressors in the form of osmotic, acidic, alkaline, cold, heat, envelope, and oxidative stress were all found to significantly increase the resistance levels of *Serratia* sp. HRI to the QAC-disinfectant Virukill®, indicating a strong role of the stress response in susceptibility and resistance to QAC disinfectants.

Author Contributions: Conceptualisation, S.M. and R.R.B.; methodology, S.M.; software, S.M.; validation, S.M. and R.R.B.; formal analysis, S.M.; investigation, S.M.; resources, R.R.B.; data curation, S.M.; writing—original draft preparation, S.M.; writing—review and editing, S.M. and R.R.B.; visualisation, S.M.; supervision, R.R.B.; project administration, R.R.B.; funding acquisition, R.R.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: All data will be made available on request.

Acknowledgments: The authors would like to acknowledge the Centre for Proteomic and Genomic

Research (CPGR) in Cape Town South Africa for technical support.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A. RNA-Seq Methodology

Appendix A.1. Ribosomal Depletion

Bacterial ribosomal RNA was depleted using the Invitrogen RiboMinus Bacteria module according to the manufacturer's instructions using 5 µg of each sample. The resulting ribosomal-depleted RNA was concentrated using the Invitrogen RiboMinus Concentration Module.

Appendix A.2. RNA-Seq Library Preparation and QC

The ribosomal depleted RNA was used for Illumina library preparation using the NEXTflex Rapid Directional RNA-Seq Kit according to the manufacturer's instructions. The ribosomal depleted RNA was fragmented and converted to single-stranded cDNA, followed by second-strand synthesis using dUTP. The cDNA was purified using Agencourt AMPure XP magnetic beads, followed by adenylation and adapter ligation. The NEXTflex RNA-Seq barcodes assigned to each sample are listed in Table 2 in the Results Section. The adapter-ligated cDNA was purified using Agencourt AMPure XP magnetic beads, treated with Uracil DNA Glycosylase to maintain strand information, and amplified by PCR for 15 cycles. Libraries were purified using Agencourt AMPure XP magnetic beads. The purified RNA-seq libraries were quantified using the Qubit 1X dsDNA HS Assay Kit. The library size distribution for each library was determined using the Agilent TapeStation D1000 assay (D1000 ScreenTape (5067–5582) with D1000 Reagents (5067–5583)).

Appendix A.3. RNA-Seq Library Pooling

The RNA-seq libraries were subsequently pooled in equimolar amounts for sequencing. The average fragment size for the pooled library was determined using the Agilent Bioanalyzer HS DNA and the region analysis function of the Bioanalyzer software available at <https://www.agilent.com.cn/zh-cn/product/automated-electrophoresis/bioanalyzer-systems/bioanalyzer-software/2100-expert-software-228259>. The concentration of adaptor-ligated DNA molecules was confirmed by qPCR using the KAPA Library Quantification Kit for Illumina Platforms on the QuantStudio 12K Flex System. A library dilution series of 1:10,000, 1:50,000, 1:250,000, and 1:1,250,000 was quantified in triplicate. The size-adjusted library concentration was calculated using the KAPA Library Quantification Kit (Illumina Platforms) Data Analysis Template (v4.14).

Appendix A.4. RNA-Seq Library Sequencing

The RNA-Seq pooled libraries were denatured with 0.2 N NaOH, diluted to 1.5 pM and combined with the denatured PhiX positive control at a spike-in concentration of 1% v/v for the NGS run according to the standard Illumina protocol. The pooled library was loaded on the Illumina NextSeq 550 instrument (serial no. NB552161) and sequenced using a NextSeq 500/550 Mid Output Kit v2.5 (150 Cycles) consisting of Mid Output Flow Cell Cartridge v2.5; Buffer Cartridge v2; Mid Output Reagent Cartridge v2 150 cycles; Accessory Box v2. The sequencer was programmed to perform a paired-end, single-indexed 2 × 76 cycle sequencing run.

Table A1. Raw expression data for the top 10 differentially expressed genes in each condition depicted in Tables 1– 3 in the main text.

Gene	Gene_Id	Logfc	LogCPM	p Value	FDR	Target Accession
Ddac Treated Cells						
273	TRINITY_DN37_c0_g2_i1	3.338479862	8.77854879	4.08442595345796	1.07511666306 sp P24169 DCOS_ECOLI	
88	TRINITY_DN7_c1_g1_i1	3.383593142	12.1530789	7.3375008818462e-145	5.99173651556 sp Q6CZC7 ILVD_PECAS	
86	TRINITY_DN7_c1_g1_i19	3.388009874	13.3162348	1.96689358672974	1.64349968770 sp Q667L9 METN2_YERPS	
855	TRINITY_DN491_c0_g1_i1	3.395842945	4.40399164	1.86084001074269	1.56397617744 sp P45512 DHAR_CITFR	
1339	TRINITY_DN295_c1_g1_i1	3.411016631	4.00999159	1.53490618476127	8.23736806847 sp P67144 YHGN_ECO57	
821	TRINITY_DN491_c0_g1_i3	3.416445822	4.45791305	1.29004756633291	1.12914516585 sp P45512 DHAR_CITFR	
625	TRINITY_DN252_9_c7_g1_i1	3.621848718	6.19829961	1.2526413477177e-29	1.44023691595 sp P0AAF2 POTE_ECOL6	
596	TRINITY_DN295_c0_g2_i2	4.095759575	5.47208783	1.51116121932068	1.82201418155 sp P39835 GNNTT_ECOLI	
338	TRINITY_DN491_c0_g1_i2	4.099837812	6.00630322	1.15922659711852	2.46455690144 sp P45511 GLDA_CITFR	
33	TRINITY_DN115_25_c2_g2_i1	4.954504662	9.03199451	1.13067207190838	2.46212409355 sp A8GA18 RS16_SERP5	
Virukill Treated Cells						
434	TRINITY_DN247_09_c3_g1_i2	2.991421512	5.68648701	7.36027924146609	1.21868586703 sp Q6CZY7 RS5_PECAS	
100	TRINITY_DN254_7_c0_g1_i1	3.000209471	9.32365502	1.67092668801677	1.20072791800 sp A8GA18 RS16_SERP5	
217	TRINITY_DN141_67_c2_g1_i1	3.011056270	7.29122407	1.43030955045743	4.73649973713 sp P36244 TRMD_SERMA	
193	TRINITY_DN142_16_c0_g1_i1	3.127840180	7.36443310	4.47081963553364	1.66462745600 sp A8AQJ0 RPOA_CITK8	
825	TRINITY_DN295_c0_g2_i2	3.239884046	5.47208783	1.68819224861457	1.47046660588 sp P39835 GNNTT_ECOLI	
1300	TRINITY_DN295_c1_g1_i1	3.285402370	4.00999159	3.45185497260201	1.90807921793 sp P67144 YHGN_ECO57	
541	TRINITY_DN58_c1_g1_i1	3.516503307	4.57526752	6.05126565800361	8.03072444320 sp P21865 KDPD_ECOLI	
939	TRINITY_DN177_2_c0_g1_i1	-	3.81309547	1.42066926394354	1.08721292126 sp P75783 YBIO_ECOLI	
242	TRINITY_DN221_24_c3_g1_i1	3.732494739	6.18519414	1.53956644231881	4.57162167541 sp Q6CZY7 RS5_PECAS	
4	TRINITY_DN115_25_c2_g2_i1	6.001438948	9.03199451	3.71999777537125	6.68297600345 sp A8GA18 RS16_SERP5	
DDAC versus Virukill treated cells						

1	TRINITY_DN37_	4.966479105	8.77854879	1.01802446322184	7.31552379271	sp P24169 DCOS_E
	c0_g2_i1	18382	810936	e-190	212e-187	COLI
6	TRINITY_DN252	4.904329948	6.19829961	9.51181694973129	1.13919861001	sp P0AAF2 POTE_
	9_c7_g1_i1	15666	437949	e-86	282e-82	ECOL6
55	TRINITY_DN530	3.658895633	4.61608830	5.02572658301582	6.56634022282	sp P16482 CITH_K
	_c0_g1_i1	2494	030995	e-29	758e-27	LEPN
35	TRINITY_DN26_	3.639230995	4.87512244	4.0817357585228e	8.15657024067	sp P39396 BTST_E
	c0_g2_i6	33661	34805	-33	536e-31	COLI
124	TRINITY_DN177	3.472671728	3.81309547	3.72472439000641	2.15853786020	sp P75783 YBIO_E
	2_c0_g1_i1	63828	721004	e-22	855e-20	COLI
17	TRINITY_DN491	3.376488416	6.00630322	8.39126240427505	3.54703597865	sp P45511 GLDA_
	_c0_g1_i2	20955	341409	e-41	415e-38	CITFR
9	TRINITY_DN133	3.264744320	6.83242869	2.311886813811e-	1.84591318267	sp P0A9H4 LDCI_
	85_c0_g1_i1	31549	007252	69	176e-66	ECO57
97	TRINITY_DN344	3.240170911	4.37846516	3.60158186704608	2.66814095841	sp J1H0Z7 SQWH_
	9_c4_g1_i1	53125	227309	e-24	166e-22	CLOS9
302	TRINITY_DN215	3.104364641	3.48303894	1.07323511805768	2.55373097959	sp P75793 GRE2_E
	_c0_g1_i2	62452	227334	e-14	024e-13	COLI
19	TRINITY_DN103	3.093001248	5.51101555	8.29090544058077	3.13570771031	sp Q8FAJ3 ULAA_
	8_c0_g1_i4	68128	787852	e-40	65e-37	ECOL6

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5.2. Chapter 5 Conclusions

The resistant isolate, *Serratia* sp. HRI was exposed to two QAC disinfectants, DDAC, the pure form of a QAC, and Viurkill®, a DDAC-based finished product commercially used in industry. RNA-Seq results revealed the most differentially expressed systems for Viurkill® treated samples were those associated with methionine transport and polyamine synthesis. An additional 5-fold increase in polyamine synthesis further differentiated DDAC-treated cells. Upon investigation, methionine and cysteine metabolism alter oxidative stress resistance through the pentose phosphate pathway (PPP) (Campbell *et al.* 2016; Bin, Huang and Zhou 2017). Elements of the PPP were also found to be differentially expressed in BC-treated cells in Chapter 4. The most significant differential expression for both QAC disinfectants was in polyamine synthesis. This was surprising regarding disinfectant resistance; however, literature revealed that polyamines function as primordial stress molecules (Rhee, Kim and Lee 2007).

Furthermore, polyamine-mediated mechanisms have been found to lead to stress tolerance and oxidative stress resistance (Liu *et al.* 2015; Solmi *et al.* 2023). In terms of antimicrobial susceptibility, polyamines have been found to alter susceptibility to antibiotics (Kwon and Lu 2007), and a polyamine (spermidine and spermine) efflux pump, AmvA, has been described that confers resistance to chlorhexidine and benzalkonium chloride disinfectants in *Acinetobacter baumannii* and *Escherichia coli*. Therefore, polyamine-mediated stress responses could be an overlooked mechanism of disinfectant susceptibility and resistance.

To elucidate the true impact of the stress response on QAC susceptibility and resistance, MICs were conducted for both the susceptible strain (*Serratia marcescens* ATCC 13880) and resistant isolate (*Serratia* sp. HRI). Susceptibility tests revealed significant reductions in susceptibility in the type strain and significant increases in resistance levels in *Serratia* sp. HRI

for all seven stressors tested. These results confirm that the stress response plays a major role in reducing susceptibility and increasing resistance to QAC disinfectants.

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Chapter 6: Discussion on the integrated relationship of the bacterial stress response and reduced susceptibility to Quaternary Ammonium Compounds

6.1. Introduction

The results in this dissertation had strong links to the bacterial stress response and suggests that this system could be a major player in QAC resistance. In order to understand the results obtained in this work further research was required to elucidate any links between the stress response and QAC resistance. Furthermore, thorough research was required to uncover the mechanisms behind how the stress response impacts QAC resistance. While researching this topic to understand the results obtained in this dissertation it was decided that a literature review was needed on the subject and presented as a discussion chapter in this dissertation. Future work includes submitting this literature review for publication in a peer-reviewed journal.

6.2. Literature review of results obtained in this dissertation

6.1. Abstract

Antimicrobial resistance poses a significant threat to human health, agriculture, and food processing. As antibiotics lose efficacy, disinfectants have become crucial for controlling microbial growth as a prevention method over dwindling treatment options. The COVID-19 pandemic increased the use of biocides, notably Quaternary Ammonium Compounds (QACs), leading to increased microbial resistance due to overuse and misuse. Research into microbial tolerance and resistance to QAC compounds reveals the bacterial stress responses may play an integral role in microbial susceptibility. The stress response is activated by exposure to antimicrobials, impacting susceptibility through various largely uncharacterised pathways. Recent studies indicate that the stress response is not general but tailored to specific stressors or antimicrobials. Thus, the resultant stress response induces specific resistance mechanisms such as efflux pumps and biofilm formation. Literature reveals resistance to QACs is modulated by changes in cell envelope properties, active stress responses, and metabolic shifts, but gaps remain regarding the underlying mechanisms. This review analyses the links between the QAC mode of action, the reactive stress response and the resultant resistance phenotype in bacteria. Elucidating the interplay between the stress response and antimicrobial resistance is crucial for developing effective strategies against AMR and ensuring the efficacy of biocides moving forward.

6.2. Introduction

Antimicrobial resistance is a well-known phenomenon with far-reaching impacts in many industries, including human health, agriculture, and food processing (Merchel Piovesan Pereira and Tagkopoulos 2019). In a time when antibiotics are running out, and resistance is more prominent, disinfectants and biocides are our last line of defence against microorganisms as there is no other viable alternative currently to control microbial growth (Allen *et al.* 2014; Bragg *et al.* 2018; Rello, Parisella and Perez 2019). As a result, we must safeguard our current biocides and research microbial tolerance and resistance to these products. The COVID-19 pandemic highlighted our reliance on biocides such as

disinfectants and hand sanitisers. Thus, it is troubling that resistance to these products is emerging at an alarming rate (Donaghy *et al.* 2019), accelerated by incorrect and overuse during the pandemic (Hora *et al.* 2020; Lai *et al.* 2021; Jefri *et al.* 2022).

The United States Environmental Protection Agency (EPA) approved 672 disinfectant products for use during the COVID-19 pandemic; Quaternary Ammonium Compounds (QACs) were the largest group of approved products, representing 37% of the total market (EPA 2021). Of the 247 QAC-based products approved for use during the pandemic, more than half (136 products) list benzalkonium chloride (BAC) as an active ingredient (EPA 2021). As this represents the most commonly used active ingredient in this context, research is urgently needed on this compound, its mode of action, its effect on the environment, and its implications for antimicrobial resistance.

Approximately 700 000 tonnes of QAC disinfectants are used annually and released into the environment (Martínez-Carballo *et al.* 2007; Ivanković and Hrenović 2010). A mega-manufacturer of disinfectant products reported that the production levels in May 2020 were equivalent to all production for the year 2019 (Thomas Buckley 2020). Another report analysed the global surface disinfectant market, projected to have a compounded annual growth rate of 9.1% between 2020 – 2027 (Fortune Business Insights 2020). Therefore, the number of disinfectant compounds released into the environment is expected to increase exponentially even with the end of the COVID-19 pandemic (Martínez-Carballo *et al.* 2007; Ivanković and Hrenović 2010; Thomas Buckley 2020). This is of great concern for a number of reasons. Firstly the devastating impacts on natural water systems and biodiversity in ecosystems, secondly the rise of microbial tolerance and resistance to these products, and lastly, the effect on human health (Ivanković and Hrenović 2010; Hora *et al.* 2020; Zheng, Webster and Salamova 2021; Boyce 2023). During the pandemic, when the use of QAC-based products increased, QACs were detected in the blood of individuals (Hrubec *et al.* 2021; Zheng, Webster and Salamova 2021). Furthermore, the presence of QACs affected inflammation, mitochondrial function and cholesterol synthesis in proportion to the amount detected in patients' blood (Hrubec *et al.* 2021; Zheng, Webster and Salamova 2021).

Although we are gathering more information on how QACs affect our bodies, there are many questions on how QACs affect bacterial physiology. Contradictory literature on the mode of action of QACs and the related bacterial tolerance and resistance mechanisms raises multiple questions (Rizvi and Ahammad 2022). This coupled with a lack of depth on the systemic molecular mechanisms of QAC tolerance and resistance results in a knowledge gap of the fundamental systems in bacterial physiology.

The bacterial stress response is a well-known defence mechanism against adverse conditions affecting bacterial survival (da Cruz Nizer *et al.* 2021). The stress response is activated upon antibiotic and biocide exposure as a protective measure against the bactericidal effects of antimicrobials, and recent research has shown it has a major impact on susceptibility to antimicrobials (Huang *et al.* 2021; Milani *et al.* 2021; Liang *et al.* 2023). This survival response decreases susceptibility to biocides and antibiotics by activating multiple intrinsic pathways (Dorel, Lejeune and Rodrigue 2006; Wei *et al.* 2012). Furthermore, antibiotic multidrug resistance (MDR) development is under regulation by global transcriptional activators like the Ara and Lys superfamilies and the oxidative stress regulon SoxRS (Chubiz and Rao 2011). By nature, antimicrobials such as antibiotics and biocides are stressors to the cell, and as a result, during all antibiotic and biocide treatment, the stress response is activated to some extent (Fraud and Poole 2011; Guest and Raivio 2016; Dam, Pagès and Masi 2018; da Cruz Nizer *et al.* 2021; Milani *et al.* 2021; Liang *et al.* 2023). Therefore, the stress response cannot be ignored when studying antimicrobial susceptibility, tolerance and resistance.

Interestingly, more and more research points to the stress response as a targeted mechanism, unique to the type of stressor or antimicrobial challenging the cell and not a general response. When *Listeria monocytogenes* was exposed to BAC, not all stress response genes were differentially expressed. Some genes were up-regulated (*iap*) or down-regulated (*SigB*) and others were unchanged (Kokkoni *et al.* 2021). Similarly, when *Escherichia coli* was exposed to sub-lethal levels of biocides, the oxidative stress response was induced after exposure to BAC, hydrogen peroxide and triclosan but not chlorhexidine (Ligowska-Marzeta *et al.* 2019). Research on the stress response and the impact on susceptibility in biocides is far less than that of the research into susceptibility to antibiotics; however by examining the work that has been done on antibiotics, we may be able to get an idea of how this

bacterial response plays a role in susceptibility to biocides. Additionally, analysing the stress response and its impact on antibiotic resistance could direct research into how the stress response can lead to biocide resistance. For instance, the CpxRA stress response system regulates expression of porins like ompF and ompC (Batchelor *et al.* 2005). As early as 1981, these porins were found to play a role in resistance to β -lactam antibiotics (Harder, Nikaido and Matsushashi 1981); in 2021 it was confirmed that these porins can lead to cross-resistance to both antibiotics and biocides in *E. coli* (Merchel Piovesan Pereira, Wang and Tagkopoulos 2021).

The oxidative stress response activates resistance mechanisms such as efflux pump systems and biofilm formation (da Cruz Nizer *et al.* 2021). This raises the question of how significant the impact of the stress response on resistance to biocides is. Literature exists on the stress response and how it can lead to reduced susceptibility, tolerance and resistance to antibiotics and biocides, yet it seems overlooked in the fight against antimicrobial resistance. This review aims to link the mechanism of action of QACs and the bacterial stress response; in light of this, it suggests that the stress response may be the regulator of some (if not all) biocide resistance mechanisms.

6.3. Biocide resistance

Compared to antibiotic resistance, resistance to biocides is a niche field of study. Until recently, it was assumed that resistance would be very difficult to develop due to the multiple targets of biocides. Today we know that is not the case with more biocide-resistant microorganisms isolated in several industries where these antimicrobials are used on a large scale (Heir, Sundheim and Holck 1999; Lynne *et al.* 2009; Kotb and Gafer 2015; Romero *et al.* 2017; Chen *et al.* 2020; Mc Carlie *et al.* 2020). The use of any antimicrobial, including biocides, will increase selective pressure for the evolution of resistance. However, improper and overuse of biocides can accelerate the development of resistant microorganisms (Bragg *et al.* 2014).

Run off from extensive factory and agricultural use creates a gradient of QAC concentrations environmentally (Oblak, Futoma-Kołoch and Wieczynska 2021). The variable environmental concentrations of QACs create selective pressure for resistant microorganisms and promotes the

establishment of resistant populations (Tandukar *et al.* 2013; Kim *et al.* 2018a; Obłąk, Futoma-Kołoch and Wieczńska 2021). Recent work by Kim and co-workers (2018) has shown that developing tolerance and resistance to BAC promotes the simultaneous development of antibiotic resistance, even without prior exposure. This co-evolution of resistance is because of shared mechanisms that reduce susceptibility or due to the transfer of grouped or shared resistance genes (Kim *et al.* 2018b, 2018a). This gives rise to multidrug-resistant isolates that can be resistant to a broad range of antimicrobials (Walsh *et al.* 2003; Wand *et al.* 2017).

Biocide resistance mechanisms include adaptations to the cell envelope such as altering the charge of the cell membrane, modification to LPS and lipid A residues to hinder antimicrobial adsorption and lower permeability of the outer-membrane (Lee *et al.* 2009a; Kim *et al.* 2018a). The composition of the outer-membrane can change to include more efflux pump proteins and fewer porins which biocides use for entry into the cell (Kim *et al.* 2018a). An increased mutation rate can also give rise to resistance as biocide targets can be altered (Condell *et al.* 2012). Resistance to the biocide triclosan is brought about by mutations in its target, the *fabI* gene (Heath *et al.* 1998). However, this mechanism is not common due to the multiple targets of most biocides. Biofilm formation is a well-known survival mechanism of microorganisms (Mah and O'Toole 2001; Borriello *et al.* 2004; Kim *et al.* 2009). However, it is essential to note that biofilms, like bacterial endospores, are a survival advantage and not a resistance mechanism as the biofilm needs to be formed and matured already to effect susceptibility and tolerance (Pagedar, Singh and Batish 2012; Obłąk, Futoma-Kołoch and Wieczńska 2021). This narrative is supported by a recent review of transcriptomic data which shows that biofilm-associated genes are not differentially expressed after biocide exposure in bacteria, and thus are not a response to biocides (McCarlie *et al.* 2023). The most recently discovered resistance mechanism is the degradation and metabolism of biocides by bacterial populations (Oh *et al.* 2013, 2014). This mechanism of reduced susceptibility has been found in microbial populations exposed to environmental sub-lethal levels of QAC biocides (as reviewed by Belter *et al.* 2022). Many of these mechanisms of reduced susceptibility and resistance are conferred by resistance genes and the acquisition of these resistance determinants

through horizontal gene transfer can create a resistant population (Hegstad *et al.* 2010; Ciusa *et al.* 2012; Mc Carlie, Boucher and Bragg 2020; Li and Wang 2021).

Finally, recent articles describe efflux pumps as the “main” or “primary” mechanism of reduced susceptibility to biocides for many microorganisms (Ribič *et al.* 2020; Sobhanipoor *et al.* 2021; de Morais Oliveira-Tintino *et al.* 2023). Efflux pumps can comprise a singular protein or multiple forming bridges through membranes; additionally efflux pumps can be broad spectrum such as multidrug resistance efflux pumps or specific like the QAC and SMR families (as reviewed by Chitsaz and Brown 2017). These efflux systems reduce susceptibility to antimicrobials by lowering the intracellular concentration of the antimicrobial, thereby limiting intracellular damage (Blair, Richmond and Piddock 2014). It is widely documented that efflux pumps play a major role in antimicrobial resistance with significant effects on susceptibility documented with efflux inhibitors (Lomovskaya and Watkins 2001; Zhang and Ma 2010; Wang *et al.* 2017; Ribič *et al.* 2020; de Morais Oliveira-Tintino *et al.* 2023). However, little information exists on whether the antimicrobial compounds are modified during efflux. If efflux pumps work by removing antimicrobials from inside the cell and pumping them outside, then the majority of the antimicrobial effect of biocides must be through intracellular targets. Otherwise, simply pumping out the active biocide compounds, without modifications would enhance the biocide attack from outside the cell and not affect susceptibility. If lowering the intracellular concentration of biocides by pumping them outside the cell, has a significant effect on susceptibility (which has been proven), this implies that biocides are not attacking cells from the outside but instead are attacking microbial cells through intracellular targets, in contradiction to current literature.

6.4. What is our current understanding of how BAC biocides work?

Biocides and disinfectants differ from antibiotics by how they target microorganisms. Antibiotics usually have a specific mechanism of action and a particular cellular target. This is why a single mutation in an individual gene can bring about resistance to an antibiotic (Martinez and Baquero 2000). On the other hand, the bactericidal activity of disinfectants is brought about by a cell-wide attack, targeting multiple cellular targets, systems and functions simultaneously (Wessels and Ingmer 2013).

Therefore, resistance to disinfectants is generally more difficult to develop as multiple resistance mechanisms must simultaneously be active within the cell (Kim *et al.* 2018a).

Many chemicals are used as active ingredients in disinfectants, including chlorine, glutaraldehyde-based products, quaternary ammonium compounds, ethanol-based disinfectants, formaldehydes and hydrogen peroxide. Every disinfectant has a specific mechanism of action, and in turn, a specific microbial resistance profile to counteract the attack by that disinfectant (Wessels and Ingmer 2013). Consequently, the mechanism of action and resultant resistance response for one disinfectant, can not necessarily be applied to another. By understanding the mechanism of action of a disinfectant, resistance mechanisms can be predicted. Furthermore, knowledge of the mechanism of action is required to apply and use biocides properly. For example benzalkonium chloride (BAC) is a cationic surfactant and the positive charge is needed for the microbicidal effects. If BAC were to be applied with an anionic (negatively charged) solution, it would negate the charge and the biocide would be less effective (Wessels and Ingmer 2013).

Like other QAC disinfectants, benzalkonium chloride is characterised by long alkyl chains and a charged ammonium group (Pereira and Tagkopoulos 2019). QAC activity is based on the length of the hydrophobic tail, type of side chain and number of cationic residues of the QAC molecule (Alkhalifaa *et al.* 2020). BAC is a cationic surfactant with high surface activity and can coat surfaces such as glass and stainless steel. This property facilitates adsorption and penetration of the hydrophobic chains into the membrane and prevents biofilm formation (Oblak, Futoma-Koloch and Wieczyska 2021).

The positively charged BAC chemical is attracted to the negatively charged bacterial cell wall (Ioannou, Hanlon and Denyer 2007; Wessels and Ingmer 2013). Once in contact with the bacterial cell, the long alkyl chain permeates the cell membrane, from the outside, causing physical disruption. While the charged nitrogen group remains on the surface of the cell membrane disrupting the surface charge of the cell (Wessels and Ingmer 2013). QACs insert into the outer-membrane by hydrophobic lipid-QAC interactions, which causes lipid clustering due to attraction by the positively charged QAC biocide (Alkhalifaa *et al.* 2020). The lateral diffusion of membrane lipids disrupts the fluidity of the phospholipid bilayer and interferes with hydrophobic and electrostatic interactions between membrane

lipids and proteins (Alkhalifaa *et al.* 2020). More specifically, BAC alters lipid organisation in the membrane and can solubilise membrane lipids and proteins at high concentrations (Obląg, Futoma-Kołoch and Wieczynska 2021).

After destabilising the charge and disrupting permeability of the outer-membrane, BAC enters the cell envelope in two ways, by simple adsorption through the membrane or in the form of dimers entering through outer-membrane porins (Ioannou, Hanlon and Denyer 2007). Moving through the cell envelope, BAC damages the cytoplasmic membrane, resulting in a loss in cell integrity and leakage of cell contents (Ioannou, Hanlon and Denyer 2007; Jansen *et al.* 2013; Barros, Melo and Pereira 2022). BAC has also been found to promote vacuole formation and further loss of intracellular contents (Barros, Melo and Pereira 2022). Current research on QAC-based biocides has accredited the attack on the cell envelope, the resultant loss of cell integrity, and the leakage of cell contents to the bactericidal activity of QAC-based biocides. As this attack on the cell envelope does cause cell lysis in several microorganisms, little research has been done to identify any other intracellular targets that could be responsible for the bactericidal activity of these biocides.

Recently, Barros and co-workers (2022) found that in addition BAC reduces metabolic activity of cells by 82% and decrease levels of ATP by up to 99% in *Pseudomonas fluorescens*. However, it remains largely unknown how BAC significantly impacts the metabolism of cells. A fluorescently labelled BAC-like compound called NBD-DDA was developed to elucidate more about the MOA of QAC compounds and the tolerance and resistance mechanisms (Nordholt *et al.* 2022). This work showed that the NBD-DDA compound localised at the cell envelope, as expected, however, tolerance to this compound was brought about by reduced cellular accumulation (Nordholt *et al.* 2022). Specifically, TolC, a well-characterised efflux pump was responsible for pumping out the compound and lowering the intracellular accumulation (Nordholt *et al.* 2022). This suggests that intracellular concentrations impacted susceptibility to this BAC-like biocide and therefore, the biocidal activity, at least in part, is active from within the cell and not from outside. This work also highlighted that there are still questions around how efflux contributes to reduced susceptibility, despite this being the most well-characterised mechanism of biocide tolerance and resistance (Nordholt *et al.* 2022).

A rising number of recent research articles point to intracellular targets as an underestimated MOA for QACs and that these targets could be as important in the biocidal activity of these biocides (Milani *et al.* 2021; Barros, Melo and Pereira 2022; Nordholt *et al.* 2022; McCarlie *et al.* 2023).

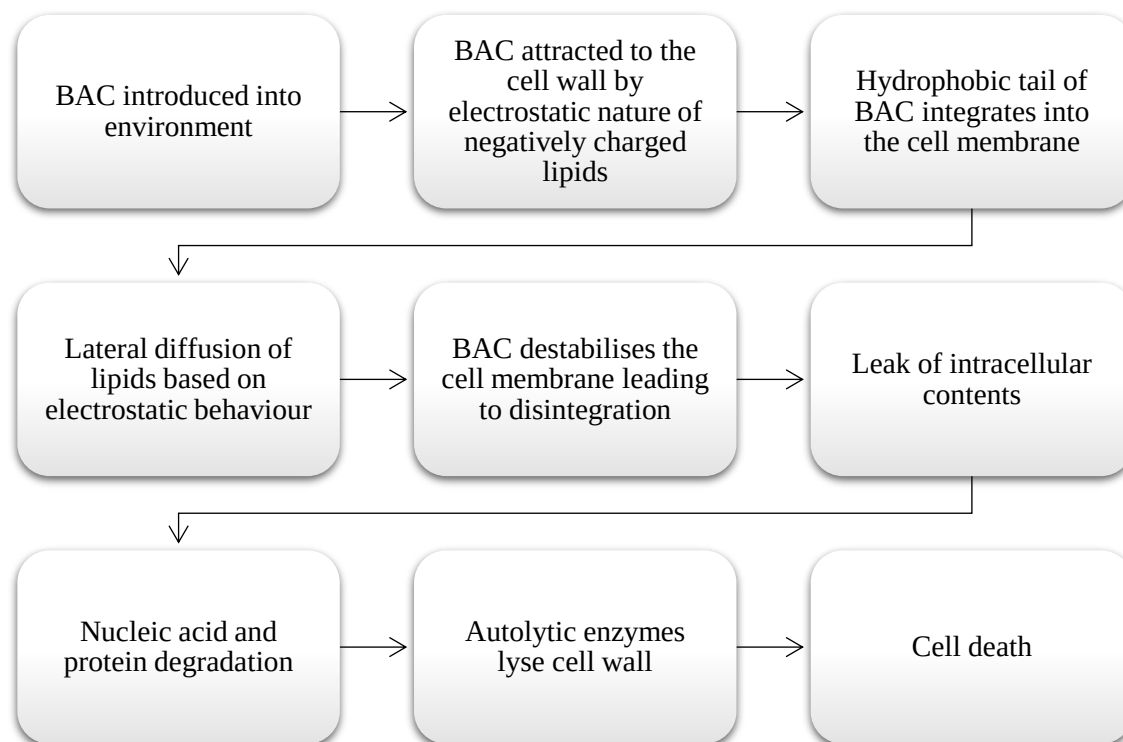


Figure 6.2 Proposed mechanism of action of benzalkonium chloride on microbial cells.

Additionally, it is important to note that if QAC-biocides do have major intracellular targets, this suggests that microorganisms may have as yet uncharacterised resistance mechanisms to protect against the intracellular portion of the attack. These intracellular resistance mechanisms are largely unknown.

6.5. Cellular response to stress

The microbicidal effect of disinfectants like BAC is brought about by an attack on many targets within the microorganism (Wessels and Ingmer 2013; Knauf *et al.* 2018; Alkhalifaa *et al.* 2020; Barros, Melo and Pereira 2022). Unlike antibiotics that often have a single target, disinfectants disrupt several targets and systems within the microbial cell. As a consequence of this far-reaching attack, multiple protective stress response pathways are triggered to reduce susceptibility and increase the chance of cell survival.

The stress response initiates multiple mechanisms that reduce the damage caused to a cell by a harsh environment or antimicrobials (NicAogáin and O'Byrne 2016). The stress response comprises a multitude of regulators that work by altering gene expression, therefore adjusting cell physiology and activating adaptive and protective responses (Poole 2012). The bacterial stress response can be activated when a cell is exposed to stressors such as nutrient limitation, oxidative stress, envelope stress, heat stress, cold stress, osmotic stress, and a general stress response that is activated when the cell is in adverse conditions (as reviewed by Poole 2012). The bactericidal effect of BAC is primarily by damaging the cell envelope and causing oxidative stress, thereby disrupting cell functions (Barros, Melo and Pereira 2022). Therefore, exposure to BAC and other QAC disinfectants will most likely trigger the envelope, oxidative and general stress responses. However, as the main function of the heat stress response is protection of the proteome (Davis, Chen and Tai 1986), damage and disruption of proteostasis may trigger this stress response as well. Finally, the environmental stress response is activated by limitations in nutrients such as iron, phosphate, fatty acid, divalent cation and carbon levels as well as cold, light and low pH (Poole 2012). This stress response network brings about a reduction in growth rate and outer membrane fortification which are known to reduce susceptibility to various antimicrobials including QAC disinfectants and BAC (Kusser and Ishiguro 1987; Vinella *et al.* 1992; Rodionov and Ishiguro 1995; Wu, Long and Xie 2010; NicAogáin and O'Byrne 2016). For these reasons, both the “temperature-dependent” and environmental stress responses will be analysed in this review as there is evidence they may have important roles to play in reducing susceptibility to BAC (Davis, Chen and Tai 1986; Vinella *et al.* 1992; Wu, Long and Xie 2010).

6.4.1 Envelope stress response

Any disruption to the cell envelope triggers several synergistic defensive responses to restore proper functioning. Antibiotics, biocides, glycopeptides, bacteriocins and bacteriophages all trigger the envelope stress response which activates defences such as efflux, reduced uptake, proteolysis and respiration changes to reduce the intracellular concentrations of these stressors (Guest and Raivio 2016). Two components systems (TCS) and sigma-factors activate the envelope stress response. In *E. coli* the envelope stress response is mediated by sigma-factor RpoE (σ^E) (Ades 2004), TCS CpxRA (Dorel,

Lejeune and Rodrigue 2006) and, BaeSR (Raffa and Raivio 2002). These TCS and sigma-factors regulate, among other things, multidrug efflux systems. The envelope stress response mediates induction of the MgrA regulator responsible for controlling expression of over 300 genes in *Staphylococcus aureus* including Major facilitator Superfamily (MFS) multidrug efflux pumps NorA, NorB and Tet38 (Chen *et al.* 2006). In *Pseudomonas aeruginosa* the MexR repressor of the well-known MexAB-OprM efflux system is mediated by the envelope stress response (Chen *et al.* 2008), and MexXY-OprM multidrug efflux pump is activated by the stress inducible PA5471 gene product (Ades 2004; Chang *et al.* 2005).

In addition to regulating multidrug efflux pumps, TCS maintain cell envelope integrity and thus decrease susceptibility to a broad range of antimicrobials (Coornaert *et al.* 2010; Huang *et al.* 2021). Several TCS have been characterised as part of the envelope stress response, some have simple but effective mechanisms of cell protection whereas others have wide knock-on effects. The AmgRS TCS is activated by envelope stress and regulates membrane transporters and protease genes which are often targets for antibiotics (Lee *et al.* 2009b). *E. coli* mutants deficient in the AmgRS TCS displayed increased susceptibility to aminoglycoside and β -lactam antibiotics and reduced virulence, even in a biofilm (Lee *et al.* 2009b). This study not only showed that the AmgRS TCS is important in antibiotic resistance but Lee and co-workers (2009b), suggest that new antimicrobials targeting these TCS can be combined with current antibiotics to increase their efficacy. The Rcs phosphorelay system is a relatively well-characterised TCS activated by envelope stress (Erickson and Detweiler 2006). Rcs-deficient *Salmonella* species mutants depicted increased sensitivity to β -lactam antibiotics and an increase in Rcs-phosphorelay activity has been linked to increased resistance to polymyxin B, a cationic antimicrobial peptide such as QAC disinfectants (Detweiler *et al.* 2003; Erickson and Detweiler 2006). In *E. coli* the CpxRA TCS reduces susceptibility to β -lactam antibiotics, aminoglycosides, cationic antimicrobial peptides, kanamycin and novobiocin (Hirakawa *et al.* 2003a). This TCS regulates multidrug efflux systems, peptidoglycan amidase genes and membrane porins OmpF and OmpC implicated in antibiotic and QAC biocide resistance (Jaffé, Chabbert and Derlot 1983; Mileykovskaya and Dowhan 1997; Hirakawa *et al.* 2003a, 2003b; Nishino *et al.* 2010; Weatherspoon-Griffin *et al.*

2011; Kim *et al.* 2018a). The additive effect of these resistance mechanisms reduces susceptibility to a wide range of antimicrobials.

Sigma-factors have far-reaching effects and can work directly to bring about change or indirectly by regulating a TCS. After exposure of *P. aeruginosa* to BAC, an *algU*-encoded envelope stress response sigma factor induced the multidrug efflux operon *MexCD-OprJ*, acting directly to reduce susceptibility to BAC (Morita *et al.* 2003; Fraud *et al.* 2008). In contrast, the RpoE sigma-factor acts indirectly by regulating the PhoPG TCS (Coornaert *et al.* 2010). When activated by RpoE, the PhoPG TCS induces lipopolysaccharide (LPS) and lipid A modification and differential porin expression to bring about resistance to cationic antimicrobial peptides, vancomycin, bacitracin, penicillin and nalidixic acid in *E. coli*, *Salmonella* Typhimurium and *Corynebacterium glutamicum* (Jaffé, Chabbert and Derlot 1983; Hirakawa *et al.* 2003a, 2003b; Coornaert *et al.* 2010).

6.4.2 Oxidative stress response

Reactive oxygen species (ROS) are by-products of aerobic growth and are harmful to the bacterial cell (Wessels and Ingmer 2013; Knauf *et al.* 2018). As a result, microorganisms have developed complex antioxidant mechanisms as a protective response (Dwyer, Kohanski and Collins 2009). Many disinfectants disrupt the oxidative state of the cell as part of their bactericidal activity, therefore, an effective antioxidant protective strategy reduces susceptibility and leads to resistance to a variety of antimicrobials (Dwyer, Kohanski and Collins 2009; Seifart Gomes *et al.* 2011; Li *et al.* 2019). The bacterial oxidative stress response includes the redox response, ROS-mediated and a nitrogen-species mediated response (as reviewed by Poole 2012).

SoxR and OxyR are the major sensors regulating oxidative stress response (da Cruz Nizer *et al.* 2021). OxyR regulates around 122 genes in response to oxidative stress impacting DNA repair, iron metabolism, anaerobic and aerobic respiration, protein synthesis, cell wall synthesis and transport of small molecules (Wei *et al.* 2012; da Cruz Nizer *et al.* 2021). OxyR, OhrR, OspR and IScR, are major regulators of the oxidative stress response (da Cruz Nizer *et al.* 2021). Their direct impact on antimicrobial resistance can be seen in the regulation of catalase A (*katA*) involved in detoxification

and the post-transcriptional modifications of oligoribonuclease (orn) which confers bacterial resistance and tolerance to aminoglycoside, β -lactam, and ciprofloxacin antibiotics (Xia *et al.* 2019). Catalase A is critical for virulence in *Pseudomonas aeruginosa* and regulated by OxyR in response to hydrogen peroxide, associated with biofilm peroxide resistance, and reduced susceptibility to nitric oxide (Lee *et al.* 2005; Shin, Choi and Cho 2008; Heo *et al.* 2010; Wei *et al.* 2012; Su *et al.* 2014; Xia *et al.* 2019).

SoxRS regulates antimicrobial resistance strategies including multidrug efflux systems, hindered disinfectant adsorption and fortifying the permeability barrier of the outer-membrane (Pomposiello, Bennik and Demple 2001; Lu, Bentley and Rao 2003; Lee *et al.* 2009a; Gu and Imlay 2011; da Cruz Nizer *et al.* 2021). SoxRS is activated by redox-cycling agents (such as paraquats) and in *E. coli*, *Salmonella* species, and *Pseudomonas* species SoxRS positively regulates multidrug efflux systems *acrAB*, *mexGHI-ompD*, *mexAB-oprM*, *mexR*, *mexCD-oprJ*, *mexEF-oprN*, and *mexXY* (Lu, Bentley and Rao 2003; Nikaido *et al.* 2011; da Cruz Nizer *et al.* 2021). Overall, the multidrug efflux pumps regulated by the oxidative stress response system have been directly linked to resistance to aminoglycoside, quinolone, tetracycline, macrolide, fluroquinolone, trimethoprim, β -lactam, and chloramphenicol antibiotics (Köhler *et al.* 1997; Masuda *et al.* 2000; Chen *et al.* 2008; Fraud and Poole 2011; Laborda *et al.* 2019; Pang *et al.* 2019).

In addition, cell envelope modifications are regulated by SoxRS such as OmpF translation, which decreases antimicrobial uptake into the cell. This, together with phosphorylation of the core heptose residues of LPS, strengthens the outer-membrane's barrier function (Lee *et al.* 2009a; Gu and Imlay 2011). After activation by an antimicrobial attack the SoxRS response alone is responsible for reducing susceptibility to antimicrobials by three synergistic strategies functioning simultaneously (Lu, Bentley and Rao 2003; Hartog *et al.* 2008; Dwyer, Kohanski and Collins 2009; Nikaido *et al.* 2011).

This direct correlation suggests that the oxidative stress response is the activator for systems responsible for antibiotic resistance. Interestingly, after *E. coli* is adapted to BAC for prolonged periods, the SoxRS activity decreases, together with the majority of stress response systems (Forbes *et al.* 2019). Therefore, the stress response may be an initial reaction to activate the rapid resistance mechanisms within a cell. This may allow for cell survival initially until long-term tolerance mechanisms are in place.

6.4.3. General stress response

An activation of the general bacterial stress response induces several downstream processes including the SOS response, an increase in mutagenesis and biofilm formation (Beaber, Hochhut and Waldor 2004; Miller *et al.* 2004; Mulcahy and Lewenza 2011). The *rpoS* gene is a well-characterised component of the overarching stress response of bacteria and is known to mediate the formation of persister cells (Dörr, Vulić and Lewis 2010), as well as a greater rate of mutagenesis to increase the chance of a favourable mutation (Galhardo, Hastings and Rosenberg 2007; Frisch *et al.* 2010). In addition to the formation of persister cells and increased mutagenesis, the general stress response reduces susceptibility through TCS (Shoji *et al.* 2011; Fernández *et al.* 2012). In *S. aureus* and *P. aeruginosa* mutants, disruptions to the ClpXP system showed higher levels of susceptibility to vancomycin and ciprofloxacin antibiotics (Shoji *et al.* 2011; Fernández *et al.* 2012).

The SOS response is a major component of any protective stress response and has far-reaching effects within the bacterial cell. Recacha and co-workers (2017) restored sensitivity to quinolone antibiotics by disrupting the SOS response in mutant *E. coli* strains. Not only was resistance reversed but MIC levels fell by up to 15-fold. This drastic reduction in resistance is hypothesised to be linked to a disruption of multiple levels of quinolone resistance, due to a knock-on effect and suppression of a multitude of resistance genes responsible for numerous resistance mechanisms after disruption of the SOS response (Recacha *et al.* 2017, 2021). Miller and co-workers (2004) found that exposure of *E. coli* to a β -lactam antibiotic activated the SOS response, which induced a halt of cell division and resistance to β -lactams, attributable to the stop in cell division. Additionally, the SOS response can induce both the acquisition and expression of antimicrobial resistance genes on mobile genetic elements (Miller *et al.* 2004; Mohanraj and Mandal 2022). Recently it was discovered that in *Vibrio cholerae* exposure to tetracycline, ciprofloxacin and azithromycin antibiotics all individually activated the SOS stress response (Mohanraj and Mandal 2022). Once the SOS response was activated, the transfer of SXT (sulfamethoxazole and trimethoprim) integrative conjugative mobile element (ICE) was induced together with the CTX prophage, which carries the *ctxAB* gene encoding the cholera toxin and virulence factor (Quinones, Kimsey and Waldor 2005; Mohanraj and Mandal 2022). It is not only resistance genes

on ICE that are affected by the SOS system but induction of mobilisation and expression by the SOS response have also been recorded for plasmid-borne resistance genes (*qnrB* quinolone resistance gene) (Da Re *et al.* 2009), integrons (Strugeon *et al.* 2016) and pathogenicity islands (SaPI_{bov1} in *Staphylococcus aureus*) (Ubeda *et al.* 2005).

In addition to playing a role in acquisition and induction of resistance genes, through mutation studies, it was discovered that the LexA regulator of the SOS response has been directly linked to biofilm formation, sporulation and resistance to metronidazole in *Clostridioides difficile* (previously *Clostridium*) (Walter *et al.* 2015). *E. coli* mutants with disrupted *rpoS* genes displayed abnormal energy metabolism, motility and could not form mature biofilms (Ito *et al.* 2008). Additionally the PhoPQ TCS and *rpoS* genes activated by the stress response system mediate biofilm formation by altering expression of biofilm regulatory genes such as *retS* (Mulcahy and Lewenza 2011; Wang *et al.* 2011).

The alarmone (p)ppGpp is a second messenger molecule activated by the stress response. It is responsible for cellular changes that decrease susceptibility to various antimicrobials (Wu, Long and Xie 2010). This second messenger molecule binds to RNA polymerase and alters the expression of various genes creating systemic and cell-wide shifts in metabolism (Syal, Rs and Reddy 2021). More specifically, when a cell is experiencing oxidative stress, (p)ppGpp leads to an up-regulation in stress response genes and down-regulates genes crucial for growth and metabolism, ultimately leading to a decreased growth rate and reduced susceptibility (Syal, Rs and Reddy 2021). Interestingly, further study has revealed that the differential expression brought about by (p)ppGpp, differs depending on cellular conditions. Syal and co-workers (2021) propose that the effect of (p)ppGpp is unique to the specific stressor and the stress response initiated by the bacterial cell differs depending on the specific environmental conditions and stressor.

6.4.4. “Temperature-induced” stress response

Literature suggests the function of the heat stress response is to maintain proteostasis, however this stress response is also induced by antimicrobial treatment suggesting it is not a true temperature-dependent response (Davis, Chen and Tai 1986; Ng *et al.* 2020). As the proteome is a major target for

many antimicrobials, including disinfectants, this protective response is activated after an attack by disinfectants (Davis, Chen and Tai 1986; Casey *et al.* 2014). Despite the well-known mechanisms of disruption to the proteome by antimicrobials, very little information exists on the heat stress bacterial response to maintain proteostasis (Ng *et al.* 2020). Tetracycline, along with several antibiotic families, target ribosomes and translation in their mechanism of action (Chopra and Roberts 2001). Ng and co-workers (2020) found that when exposed to a higher temperature *Mycobacterium abscessus* exhibited a higher level of resistance to tigecycline (a tetracycline antibiotic). The mechanism of this resistance is attributed to the overexpression of the a SigH ortholog, chaperones and oxidoreductases after activation of the heat stress response, this lead to induction of differential expression of ribosomal-associated genes, the target for the tetracycline antibiotic (Ng *et al.* 2020). The activation of any protective response to maintain proteostasis will counteract the mechanism of action of antimicrobials which aim to disrupt the proteome. This includes protein chaperones such as the ClpL heat shock chaperone which has been linked to β -lactam antibiotic resistance in *Streptococcus pneumoniae* (Tran *et al.* 2011). Literature on the effect of the heat induced stress response during biocide exposure is severely limited. *Listeria monocytogenes* exposed to Benzethonium chloride triggered a 9-fold up-regulation of cold shock genes (*cspCD*) together with several genes aligned with the rsbRA component of the piezosome/stressosome (Casey *et al.* 2014). The impact of the differential expression of these systems on susceptibility to antibiotics has been studied, however their effect on biocide susceptibility is not well-understood yet.

6.4.5. Environmental stress response

A wide range of environmental stressors can activate different aspects of the bacterial stress response (Hartog *et al.* 2008; Nguyen *et al.* 2011). One stressor is nutrient limitation which results in reduced growth rate and has long been associated with a lower level of susceptibility to a wide range of antimicrobials including the disinfectant benzalkonium chloride (Eng *et al.* 1991; Kim *et al.* 2018a; Forbes *et al.* 2019). Current knowledge on the nutrient limitation stress response suggests it is activated by a limitation in iron, phosphate, fatty acids, carbon and low levels of available divalent cations (Mg^{2+}) (as reviewed by Poole 2012b). The activation of the nutrient-limited stress response results in resistance

to a variety of antimicrobials including β -lactam, quinolone and tetracycline antibiotics by a slower growth rate, a halt in cell division and modifications to the cell envelope (Eng *et al.* 1991; Vinella *et al.* 1992; Rodionov and Ishiguro 1995; Proctor *et al.* 2006; Richards, Strandberg and Gunn 2010; Wu, Long and Xie 2010; Chávez de Paz *et al.* 2012; Syal, Rs and Reddy 2021). Furthermore, changes in metabolism such as a reduced metabolic rate, brought about by the nutrient stress response, is linked to decreased susceptibility to antimicrobials in *P. aeruginosa* and *Mycobacterium tuberculosis* (Borriello *et al.* 2004; Mitchison and Coates 2004; Kim *et al.* 2009).

The stringent response brings the greatest effects of the nutrient stress system (Kusser and Ishiguro 1987; Vinella *et al.* 1992; Rodionov and Ishiguro 1995; Nguyen *et al.* 2011; Strugeon *et al.* 2016). Nutrient limitations activate the stringent response and induces the expression of (p)ppGpp. An increased level of (p)ppGpp has far-reaching effects within the cell, this includes a down-regulation of genes associated with cellular growth, an up-regulation of survival nutrient scavenger genes and transcriptional switching to bring about metabolic changes needed due to limited nutrients available (Kusser and Ishiguro 1987; Williams and Rogers 1987; Gilbert, Collier and Brown 1990; Wu, Long and Xie 2010; Strugeon *et al.* 2016; Syal, Rs and Reddy 2021). More specifically, an increase in the levels of (p)ppGpp down-regulates peptidoglycan, fatty acid and phospholipid synthesis leading to a decrease in growth rate and reduced susceptibility (Ishiguro and Ramey 1980; Heath, Jackowski and Rock 1994; Wu, Long and Xie 2010). Multiple studies have found that (p)ppGpp protects against oxidative stress and (p)ppGpp deficient cells are more susceptible to a variety of antimicrobials, specifically those that produce hydroxyl radicals as part of their mechanism of action such as biocides (Heath, Jackowski and Rock 1994; Kohanski *et al.* 2008; Nguyen *et al.* 2011).

In addition to the modifications in the cell envelope and growth stages brought about by the stringent response, limited environmental cation levels activates the PhoPQ TCS (Richards, Strandberg and Gunn 2010; Storey *et al.* 2020; Huang *et al.* 2021). The PhoPQ TCS is responsible for differential expression of several genes bringing about changes to the cell envelope that reduce adsorption of cationic antimicrobials, including positively charged QAC biocides (Richards, Strandberg and Gunn 2010; Storey *et al.* 2020; Huang *et al.* 2021). Furthermore, the PhoPQ TCS can confer cross resistance to

cationic antibiotics and biocides but only in certain conditions. Wand and co-workers (2017) found that *Klebsiella pneumoniae* isolates resistant to chlorhexidine, also exhibit resistance to the last resort antibiotic colistin. Microbial mutants and expression studies found that this resistance to both the biocide chlorhexidine and the colistin antibiotic is attributable to an up-regulation of the PhoPQ TCS (Storey *et al.* 2020). However, when susceptible isolates were exposed to the antibiotic first, resistance to colistin was exhibited but not to chlorhexidine (Storey *et al.* 2020). This work puts forward the idea that if biocide resistance develops first, then antibiotic resistance follows and not the other way around. Importantly, this shows that components of the bacterial stress response system can create a cross-resistance phenotype.

An acidic environment can also trigger parts of the stress response, a low pH in *L. monocytogenes* induces the acid tolerance response, the arginine deaminase system and the glutamate decarboxylase system that alter gene expression through sigma factors (SigB) and TCS to improve chances of survival in an acidic environment (Davis, Coote and O’Byrne 1996; Cotter, O’Reilly and Hill 2001; Ryan *et al.* 2009).

6.6. Impact of the stress response on resistance to biocides

As mentioned previously, six main mechanisms of bacterial resistance to disinfectants have been identified. These mechanisms are: 1) efflux, 2) cell envelope adaptations, 3) biofilms, 4) acquisition of resistance determinants through HGT, 5) target alteration by mutations and 6) metabolism or modification of disinfectants. Interestingly, the mechanisms induced (in part) by the stress response include efflux, cell envelope adaptations, biofilm formation, HGT of resistance genes and an increased mutation rate. The only mechanism of disinfectant resistance not influenced by the stress response is the modification or metabolism of disinfectants, which is the most poorly characterised mechanism. This suggests there may still be a link between this mechanism and the stress response that has yet to be discovered.

When analysing the stress response in more detail, it is clear that the stress response confers reduced susceptibility to biocides (Casey *et al.* 2014; Wand *et al.* 2022). However, little to no research has been

done on the stress response and its impact on biocide resistance, as most research focuses on antibiotic resistance. However, resistance mechanisms common to antibiotics and biocides can be considered, and those mechanisms unique to biocide resistance can be inferred from work done on antibiotics. For example, several multidrug resistance efflux systems are activated during the stress response including the AcrAB-TolC multidrug efflux system which has been directly linked to biocide resistance in chlorhexidine and benzalkonium chloride (Forbes *et al.* 2019; Wand *et al.* 2022). This multidrug efflux system and several others are induced by the SoxRS regulator in response to oxidative stress (Bialek-Davenet *et al.* 2011; Nikaido *et al.* 2011).

Transcriptomic and expression studies have revealed that major mechanisms of biocide resistance include adaptations of the cell envelope, such as a change in charge, modifications to LPS and lipid A, and reduced numbers of porins (Fernández-Cuenca *et al.* 2015; Kim *et al.* 2018a; Lu *et al.* 2018; Ligowska-Marzeta *et al.* 2019). These changes are brought about by several cellular systems but many are activated by the stress response. Differential porin expression and lipid A and LPS adaptations are induced by the PhoPQ TCS and stringent response mediated by (p)ppGpp (Coornaert *et al.* 2010; Wu, Long and Xie 2010; Wand *et al.* 2017; Huang *et al.* 2021; Syal, Rs and Reddy 2021). The stringent response also triggers biofilm formation together with sigma-factors which are crucial for long-term biofilm maintenance (as reviewed by Poole 2012). Lastly, the acquisition of resistance genes and a greater mutation rate is activated by the SOS stress response and the general stress response respectively (Beaber, Hochhut and Waldor 2004; Guerin *et al.* 2009; Strugeon *et al.* 2016; Mohanraj and Mandal 2022). Based on the available literature, the stress response is the switch that turns on several antimicrobial resistance mechanisms. A great deal more research is required to determine how much of an impact the stress response has on antimicrobial resistance. The stress response has been studied with a very narrow reference point. Research has focused on the function of individual sigma-factors and specific TCS, and their impact on certain resistance genes. However, many questions remain, such as, how important is the stress response to antimicrobial resistance? Specifically, does the stress response enhance antimicrobial resistance or is it responsible for the entire resistance phenotype? Another question is whether we can link the function of more resistance mechanisms to the stress response.

Little to no information exists on the stress response and biocide resistance. We can link resistance genes associated with the stress response to those known to play a role in biocide resistance but not on the direct impact of the stress response on resistance to biocides. Currently, we can infer the work on antibiotic resistance and use this as a starting point when considering resistance to biocides.

6.7. Novel antimicrobials: Targeting the stress response

If the stress response does play a significant role in biocide and antibiotic susceptibility, this could direct the design of novel antimicrobials targeting major points in the stress response. Tang and co-workers (2023) developed a novel antimicrobial peptide targeting Gram-negative bacteria's envelope stress response system. The antimicrobial peptide BING suppresses the expression of the CpxR major regulator and in turn affects multidrug efflux systems, peptidoglycan amidase genes and membrane porins OmpF and OmpC implicated in antibiotic and QAC biocide resistance (Jaffé, Chabbert and Derlot 1983; Mileykovskaya and Dowhan 1997; Hirakawa *et al.* 2003a, 2003b; Nishino *et al.* 2010; Weatherspoon-Griffin *et al.* 2011; Kim *et al.* 2018a). BING also suppresses flagellar and chemotaxis gene expression associated with antibiotic evasion and pathogenicity (Guttenplan and Kearns 2013; Tang *et al.* 2023). The development of BING is the direct application of this research into the bacteria stress response and its effect on susceptibility; this peptide has been created to work synergistically with current antibiotics (ampicillin, amoxicillin, novobiocin) in the time of the antibiotic resistance crisis (Tang *et al.* 2023).

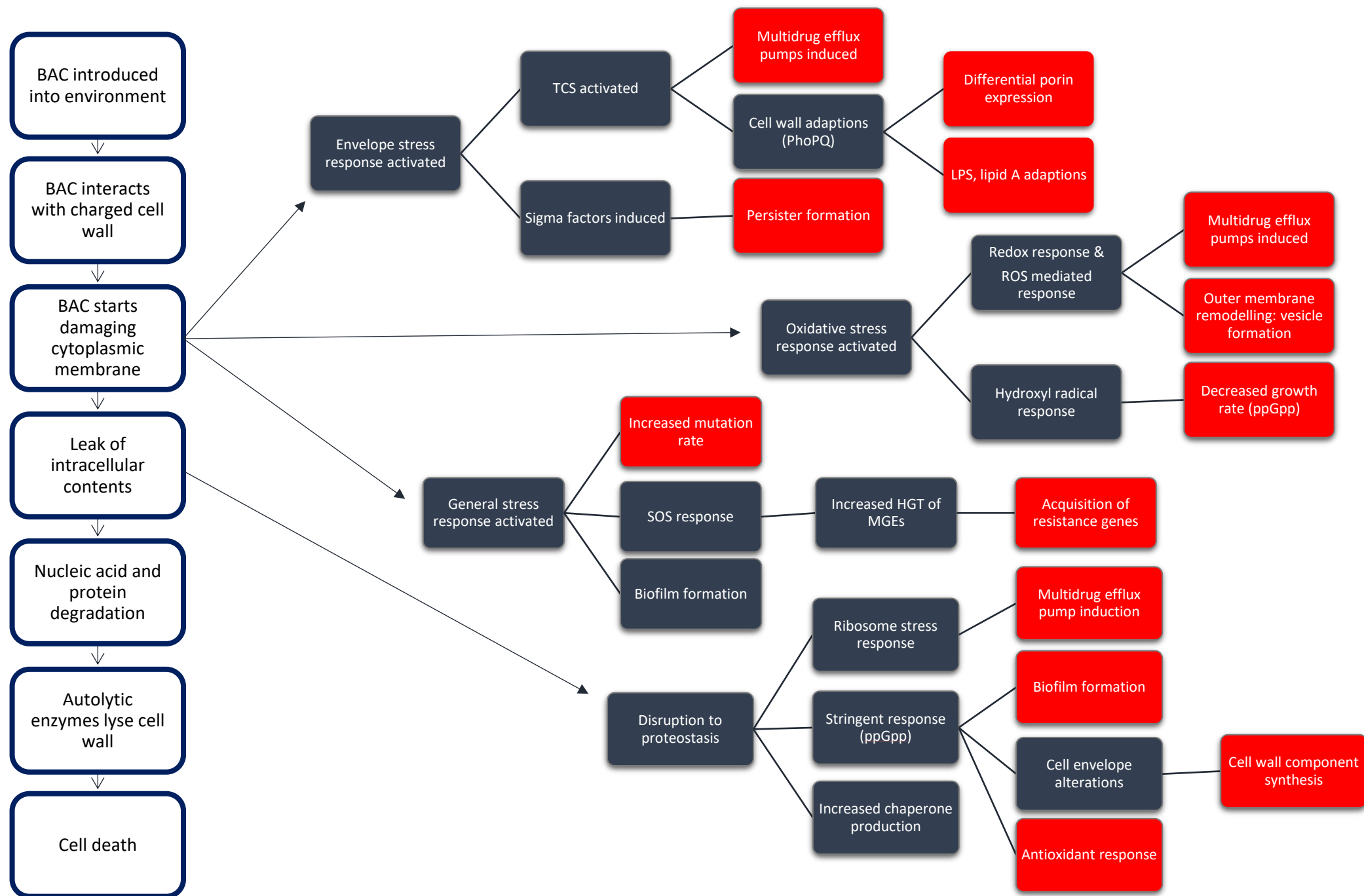


Figure 6.2 Proposed bacterial stress response activated by exposure to benzalkonium chloride. White blocks: BC MOA, Blue blocks: Triggerred stress response; Red blocks: Disinfectat resistance mechanism activated.

6.8. Conclusions

Resistance mechanisms of BAC and other QAC-based compounds focus on lowering intracellular concentrations as the most effective resistance strategy (for example through efflux pumps and porin changes). This suggests that most of the damage done by QAC-compounds is from inside the cell. If this is the case, which literature supports, then the true MOA of QAC-compounds needs urgent attention and intracellular targets must be elucidated. More research is required to uncover resistance mechanisms capable of defence of these intracellular targets.

Current proposed mechanisms of resistance to BAC include changes to the cell envelope charge and composition (including efflux proteins), target alteration through mutations, biofilm formation, acquisition of resistance genes through HGT and most recently described degradation or metabolism of BAC (Ciusa *et al.* 2012; Bragg *et al.* 2014; Oh *et al.* 2014; Ertekin, Konstantinidis and Tezel 2017; McCarlie, Boucher and Bragg 2020). However, differential expression profiles do not completely align with current proposed mechanisms and point to different responses to BAC to reduce susceptibility. Based on differential expression profiles, three main areas where cell changes are recorded in response to BAC, these are alteration to the cell envelope in terms of charge and composition, an active stress response and a metabolism shift to protect the proteome (Kim *et al.* 2018a; Merchel Piovesan Pereira, Wang and Tagkopoulos 2020; McCarlie *et al.* 2023). Further analysis of the stress response has revealed that this response regulates several resistance mechanisms including efflux pumps, cell envelope adaptations, biofilm formation, HGT of resistance genes, increased mutation rate and protection of the proteome (Batchelor *et al.* 2005; Dam, Pagès and Masi 2018; Davis and Brown 2020; da Cruz Nizer *et al.* 2021; Liang *et al.* 2023). When looking at the stress response, all current proposed mechanisms of resistance to BAC are induced except metabolism and modification of BAC. This mechanism also happens to be the least characterised resistance mechanism and may still be linked to the stress response or brought about by differential expression.

Based on the numerous differential expression studies, gene knockouts and susceptibility articles reviewed here, the stress response is critical to biocide tolerance and resistance. Literature points to the stress response as the activator of almost all known biocide resistance mechanisms. Therefore, the stress response cannot be underestimated in its far-reaching impact on antimicrobial resistance.

Based on current trends in literature, this work puts forward the idea that the stress response could be the switch that turns on several well-established antimicrobial tolerance and resistance mechanisms. This notion is reinforced by several studies showing that bacteria deficient in certain stress response systems, have higher susceptibility to antimicrobials and resistance can be reversed altogether (Heath, Jackowski and Rock 1994; Detweiler *et al.* 2003; Erickson and Detweiler 2006; Kohanski *et al.* 2008; Lee *et al.* 2009b; Nguyen *et al.* 2011). More work will need to be done to enhance our understanding of biocide MOA and how significant the stress response is in biocide resistance.

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Chapter 7: General summary and conclusions

Antimicrobial resistance is a world-wide crisis with deadly implications. The focus of most antimicrobial resistance research has been on microbial resistance to antibiotics. However, with fewer and fewer new antibiotics entering the market, we may be approaching a post-antibiotic era. Currently the only viable alternative to antibiotics to control microbial growth is good biosecurity and disinfection practices. These chemicals are vital in agriculture for disease prevention and maintaining export standards. Disinfectants are crucial to maintain hygiene and safety standards in the beverage and food industries therefore safeguarding food security and preventing food-borne illness. Disinfectants are also important preservatives in cosmetic and pharmaceutical products preventing infection. Finally, disinfectant products are critical in infection prevention and control in medical environments, directly responsible for reducing morbidity and mortality rates of hospital-acquired infections.

The antibiotic resistance crisis can be seen as a foreshadowing to a larger issue which would be a disinfectant resistance crisis. Therefore, more research is required into resistance to disinfectants to safeguard our current disinfectants and direct future development and design of disinfectant products. Disinfectant resistant isolates, such as *Serratia* sp. HRI, are valuable resources to uncover the mechanisms of microbial resistance to disinfectants.

This dissertation aimed to identify mechanisms involved in high levels of QAC resistance in *Serratia* sp. HRI. The hypothesis for this work is that for *Serratia* sp. HRI there exists a combination of mechanisms, both known and well-characterised and perhaps novel, uncharacterised mechanisms as well. The first objective was to determine if cell morphology and biofilm formation of *Serratia* sp. HRI were affected by QAC-treatment at sub-MIC levels compared to the type strain. The second objective of this study marked the move towards molecular analysis in the form of RNA-Seq to screen for novel disinfectant resistance genes by differential gene expression in *Serratia* sp. HRI when exposed to sub-MIC levels of benzalkonium chloride. Finally, the fourth objective was to build on the previous work

by analysing differential gene expression of *Serratia* sp. HRI during treatment with two additional QAC disinfectants (DDAC and Viurkill®).

In the first Chapter of this dissertation, a bacterial transcriptomic profile was created for benzalkonium chloride using all available literature on a variety of bacteria treated with BC. This transcriptomic signature was further characterised into a short-term (≤ 30 minutes) and long term (≥ 90 minutes) and compared to current mechanisms of disinfectant resistance. Little correlation was found between current mechanisms of QAC resistance and the transcriptomic response of bacteria to BC. This raised questions as to where the disconnection lies to explain the differences between current resistance mechanisms and the bacterial response to disinfectants.

Chapter 2 revealed an unexpected phenomenon with troubling implications. The hormetic effect was induced in *Serratia* sp. HRI when cultivated in the presence of sub-MIC disinfectants, significantly increasing the maximum specific growth rate. This stimulation of microbial growth could lead to increased infections in wound care, increased levels of hospital-acquired infections and impact safety standards in several industries if the disinfectants are not used correctly. This was the second time this phenomenon was observed for disinfectants and the first time in a Gram-negative bacterium (McCarlie et al., 2023; Morales-Fernández et al., 2014). The hormetic effect is hypothesised to be as a result of an overstimulated stress response (Morales-Fernández et al., 2014; Tauffenberger et al., 2019).

In the third Chapter, changes in cell morphology and biofilm formation in *Serratia* sp. HRI were revealed after treatment with sub-MIC levels of disinfectants. Interestingly, no biofilm formation was observed after 24-hour exposure to the QAC disinfectants for the resistant isolate. Although, this did align with DGE profiles in Chapter 1 which did not include any differential expression in biofilm formation genes when bacteria were exposed to BC. Instead, a multitude of cell nodules or outer membrane vesicles were found for the resistant isolate, indicating that these OMVs may contribute to the high levels of QAC resistance. OMVs have been found to reduce susceptibility to antibiotics by acting as a physical barrier and containing β -lactamases (Kim et al., 2018; Roszkowiak et al., 2019).

Thus, OMVs seem like a plausible mechanism to reduce susceptibility to disinfectants as well. Little to no literature is available on the effect of OMVs on susceptibility or resistance to disinfectants. Although, the production of OMVs has been attributed to the stress response in *Pseudomonas aeruginosa* (Macdonald and Kuehn, 2013), the mechanism is included in Figure 6.2 in Chapter 6. In addition, atomic percentage analysis conducted in this chapter suggests that QAC disinfectants work from inside the cell and not from the outside as currently hypothesised. This is supported by the impact of efflux systems which confer resistance to QACs by reducing the intracellular concentration of these antimicrobials (as reviewed by Jaglic and Cervinkova, 2012).

Chapter 4 of this dissertation generated lists of potential novel QAC resistance genes and their activity at different time points for further study. This work identified an uncharacterised MFS pump and confirmed significant up-regulation after exposure to three different QAC disinfectants, at different levels. Additionally, a higher level of expression of the UMFS pump was observed at 20 minutes of exposure compared to 90 minutes of exposure, suggesting that efflux pumps may be an initial survival response that tapers over time.

Transcriptomic analysis in Chapter 5 revealed polyamine synthesis as the most differentially expressed system for both DDAC and Virukill® exposure in *Serratia* sp. HRI. Upon investigation, it was discovered that polyamines function as part of the stress response and have been found to confer resistance to disinfectants through polyamine-mediated efflux pumps. The differential gene expression of efflux pumps highlighted in Chapters 4 and 5 of this work is observed together with differential expression of the stress response systems. These systems could be linked in their response to QACs as the activation of multidrug efflux pumps by the stress response is well characterised as depicted in Figure 6.2 in Chapter 6. Finally, susceptibility tests confirmed the role of the stress response in reducing susceptibility to QACs in *Serratia marcescens* ATCC 13880 and significantly increasing QAC resistance levels in *Serratia* sp. HRI. These changes are brought about through a combination of mechanisms activated by the stress response, outlined in Figure 6.2 in Chapter 6. This work suggests

that the stress response is the activator of several disinfectant resistance mechanisms through differential gene expression.

The following research questions were put forward at the beginning of this work: (1) What mechanisms are responsible for the high levels of QAC resistance in *Serratia* sp. HRI? (2) Does the differential expression of resistance genes play a role in the disinfectant resistance of *Serratia* sp. HRI? (3) Are there multiple mechanisms of resistance acting simultaneously to confer the resistance phenotype in *Serratia* sp. HRI?

This work suggests that multiple mechanisms are responsible for the high levels of QAC resistance in *Serratia* sp. HRI. This dissertation suggests that biofilm formation is not a QAC resistance mechanism, and instead OMVs may play a greater role. Additionally, QAC disinfectants may work from inside the cell and not from the outside as the current hypothesis states. Overall, this work puts forward the novel hypothesis that the stress response plays a major role in resistance to disinfectants, acting as a regulator which activates disinfectant resistance mechanisms through differential gene expression.

7.1. References

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