An investigation of resistance to quaternary ammonium compound disinfectants in bacteria

By

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For my father, Mr. Arrie Cornelius Jansen for giving me the opportunity to pursue my degree and inspiring me to do what I love.

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Chapter 1
Bacterial resistance to Quaternary ammonium compounds. Literature review.

1.1. Introduction

Antibiotics have been used at sub-therapeutic levels as growth promoters in animals almost since their discovery, but the problems associated with the development and spread of antibiotic resistance have been steadily increasing since 1960s. (Feighner & Dashkevicz, 1987; Gilbert & Moore, 2005). This widespread and unrestricted use of antibiotics has led to a surge in antibiotic resistant bacterial related cases such as the increase in *Salmonella* resistant to Fluoroquinolone reported in the United States of America, Asia and Europe (Herikstad *et al*., 1997; Wiuff *et al.,* 2000; White & McDermott, 2001). The development of resistance to antibiotics is usually associated with their overuse and abuse, as well as the acquisition of genetic elements encoded within plasmids (Rao, 1998; Feinman, 1999; Georgala, 1999; Magee *et al.,* 1999, Dixon, 2000). These increases had been related to the use of Fluoroquinolone as growth promoter in animals (Herikstad *et al*., 1997; Wiuff *et al.,* 2000). The use of Fluoroquinolone as growth promoters in animal production was subsequently banned to reduce the incidence of bacterial resistance to Fluoroquinolone, but soon after, the use of therapeutic antibiotics increased to prevent and control bacterial infections in animals (Casewell *et al.,* 2003). More restrictions on the use of antibiotics have been imposed and this led to an increase in the search for possible alternatives to control bacterial diseases in animal production (Joerger, 2003). Such alternative treatment methods include bacteriocins, small antimicrobial peptides and bacteriophages (Joerger, 2003). These methods seem promising but they are still in the developmental stage and the safety applications of these methods are uncertain.

The use of disinfectants could possibly be the last line of defence for the poultry industry (Joerger, 2003; Nelson *et al.,* 2007). Quaternary ammonium compound (QAC) based disinfectants are frequently used in environments were antibiotics are used thus fuelling the concern of a link between QAC and antibiotic resistance (Hegstad *et al.,* 2010). Recent reports have shown the existence of bacteria

containing *qac* resistance genes that confer resistance against QAC (Bjorland *et al.,* 2001; 2003; Smith *et al.,* 2008; Gillings *et al.,* 2009a; b). The development of extensive resistance to disinfectants and the spread of resistance would have serious consequences for the poultry industry.

The *qac* resistance genes can be found on the same DNA elements as antibiotic resistance genes such as integrons, genetic elements capable of capturing and expressing exogenous gene cassettes of which the Class 1 integrons are the most studied (Hardwick *et al.,* 2008). Class 1 integrons are known to be widespread where they typically harbour one or more gene cassettes (mobile genetic elements) imparting resistance to a wide range of hazardous substrates (Hardwick *et al.,* 2008). They provide selective advantages relevant to environmental pressures and occur in a broader range of host organisms than had previously been assumed (Hardwick *et al.,* 2008; Gillings *et al.,* 2008 a; b; 2009 a; b). The rapid spread of Class 1 integrons has been facilitated by their location on mobile DNA elements such as plasmids and transposons coupled with their selective advantage conferred by their associated antibiotic resistance genes (Gillings *et al.,* 2008 a; b).

The following review consists of information on Quaternary ammonium compounds, the development of resistance to these compounds as well as techniques, such as real time PCR, used to identify these genes and an exciting new technology, NanoSAM that will help to visualize and give greater depth and understanding to morphological changes in cells when exposed to QAC's.

1.2. Quaternary ammonium compounds

Quaternary ammonium compound (QAC) based disinfectants play an important role in veterinary medicine, in the control of animal diseases (Bjorland *et al.,* 2005). An example of the control of bacterial diseases in poultry is the continual disinfection program, where a non-toxic, modified QAC based disinfectant has been used on a continual basis in poultry production, where a reduction in bacterial loads have been recorded (Bragg & Plumstead, 2003; Bragg, 2004). The lack of selective toxicity of disinfectants and lack of target specificity makes them different from antibiotics (Denyer & Stewart, 1998). It is very important to understand the mode of action of QAC based disinfectants and the mechanisms of bacterial resistance against such compounds particularly in the light of the pending post antibiotic era that the poultry industry is facing so as not to repeat mistakes made with antibiotics.

QACs are cationic surface active detergents widely used for the control of microorganisms in clinical and industrial environments and used in the disinfection of hard surfaces (Ioannou *et al.,* 2007). They are amphoteric surfactants and contains one quaternary nitrogen that is associated with at least one major hydrophobic substituent such as alkyl groups or substituted alkyl groups, represented with R, and an anion such as Cl or Br, represented with X (Fig 1.1) (Gilbert & Moore, 2005; Schmidt, 2003).

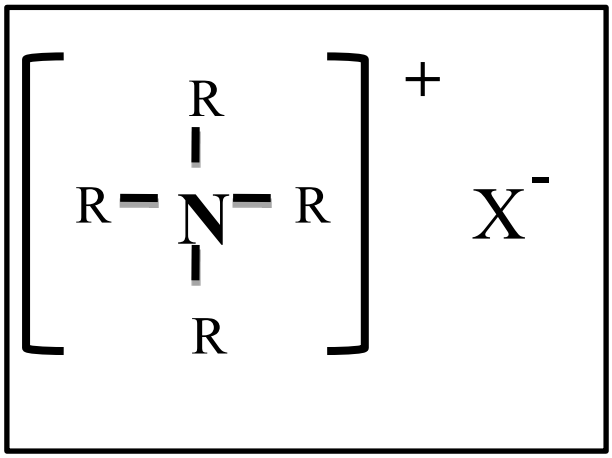

Figure 1.1. The general structure of QACs. A quaternary nitrogen atom surrounded with hydrogen atoms, alkyl groups or substituted alkyl groups represented with R and X represents an anion (Schmidt, 2003; Jacobs *et al.,* 1916a)

Jacobs and co-workers (1916b) published a paper describing the antimicrobial activity of quaternary ammonium compounds and later in 1935 it was shown that aliphatic groups with 8 – 18 carbons possesses antibacterial activity (Hegstad *et al.,* 2010). The primary target of QACs seems to be the cytoplasmic (inner) membrane of bacteria (Hegstad *et al.,* 2010). QACs are thought to adsorb to the relatively anionic bacterial cell walls, diffuse through the cell wall and binds to the cytoplasmic membrane (Hamilton, 1968; Hegstad *et al.,* 2010; Ioannou *et al.,* 2007; Sandt *et al.,* 2007). Here they possibly cause the disorganisation of cytoplasmic membrane which is thought to result in the leakage of intracellular material and ultimately causing cell death (Ioannou *et al.,* 2007). The positively charged nitrogen group interacts with the phospholipids followed by the hydrophobic tail that integrates into the hydrophobic membrane core (Ioannou *et al.*, 2007; Hegstad *et al.*, 2010). Here they cause the disorganisation of the cytoplasmic membrane resulting in the release of intracellular

molecules such as potassium ions and other intracellular low molecular weight material. QACs cause leakage of the cellular material purely because they adsorb to the cell membrane in large amounts causing damage (Ioannou *et al.*, 2007).

Bacterial cells surface carries a negative charge that is often stabilised by cations such as Ca^{2+} and Mg^{2+} (Gilbert & Moore, 2005). Cell membranes consist of proteins and lipids approximating to a bilayer where the proteins either cross (integral proteins) the bilayer or associated with a specific side of the membrane (Singer & Nicolson, 1972). The proteins associated with the cell membrane fulfil specific roles such as the maintenance of the structural integrity of the cell membrane as well as functional roles associated with metabolism such as cellular transport, biosynthesis of the cell wall and the extracellular products (Singer & Nicolson, 1972; Gilbert & Moore, 2005). Each of these proteins is surrounded by a specific phospholipid that interacts with it and influences its functionality, along with cations such as Ca^{2+} they stabilizes the lipid bilayer (Singer & Nicolson, 1972).

Many disinfectants are cationic in nature and exploit the interactions of Ca^{2+} and phospholipids with the cell membrane (Singer & Nicolson, 1972). Cationic disinfectants interact with the cell surface through their strong positive charge and hydrophobic region by integrating into the cytoplasmic membrane. The disinfectants interact initially by displacing the cations (Daoud *et al.,* 1983; Gilbert & Al-Taae, 1985). This interaction of disinfectant with the cell is enough to hinder growth.

Gilbert and Moore (2005) proposed a model for the adsorption of the QACs to the bacterial cell membrane (Fig 1.2). The positively charged quaternary nitrogen interacts with the head groups of acidic phospholipids and subsequently the hydrophobic tail integrates into the hydrophobic membrane core (Fig 1.2 b, c). At minimum growth inhibitory concentrations (MIC), the disinfectant binds firmly to anionic sites on the membrane surface, increasing the surface pressure in the exposed membrane and decreasing the membrane fluidity thus causing it to loose physiological functions such as osmoregulatory capacity and resulting in the leakage of potassium ions and protons (Figure 2d; Salt & Wiseman, 1970; Lambert & Hummond, 1973). The antibacterial concentrations used in practice are sufficient to cause the membrane to lose fluidity and cause cell death. Disinfectants form mixed micellular aggregates that solubilizes the hydrophobic membrane components (Fig 2e, f; Salton, 1951). The activity of the disinfectants are dependent on their lypophilicity (*n*-alkyl chain length) thus the nature of the disinfectant determines the

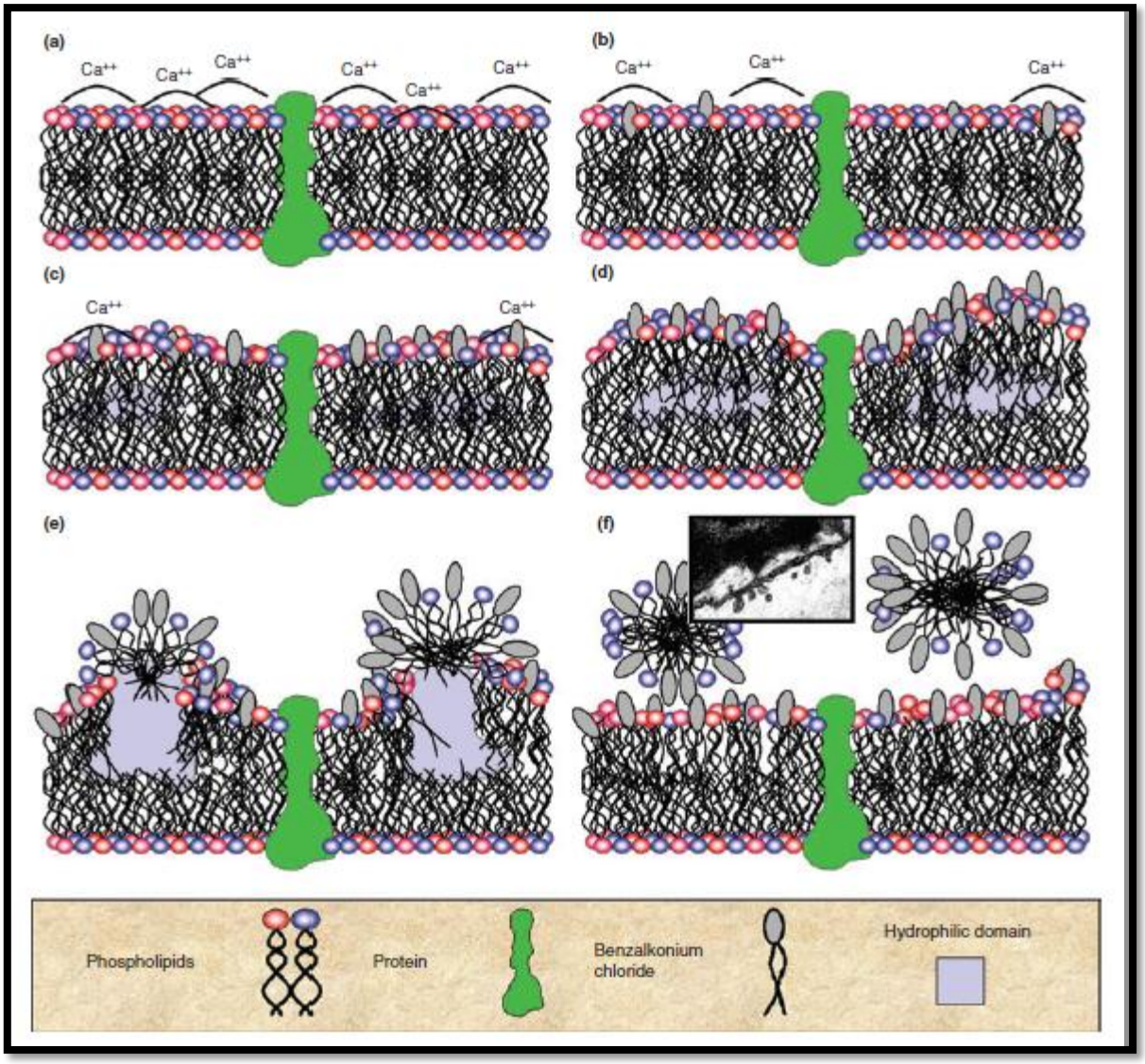

Figure 1.2. Mechanism of action of quaternary ammonium disinfectants (Gilbert & Moore, 2005).

interaction of the disinfectant with the cell membrane (Daoud *et at.,* 1983, Gilbert & Al-Taae, 1985). Daoud *et al.,* (1983) found that an alkyl chain length of *n* = 12-14 has maximum activity against gram positive bacteria and yeasts while that of *n* = 14-16 has maximum activity against gram negative bacteria. In order to maximize the spectrum of antibacterial activity, many disinfectant mixtures are blended.

1.2.1. Resistance to QACs

Bacteria have always been capable of acquiring genes that enable them to survive harsh environments (White & McDermott, 2001). The widespread use, and to a degree, the misuse of antimicrobial agents have caused selective pressure on

bacteria and one of the biggest reason for the development of resistance to these antimicrobial agents. (Sidhu *et al.,* 2002). Exposure of microorganisms to sub-MIC concentrations could result in the emergence of clones resistant to QACs (Hegstad, 2010). Disinfectants are generally used at very high concentrations but there is always the possibility that some bacteria are exposed to sub-MIC concentrations which could result in the development of resistance.

Quaternary ammonium compound based disinfectants are positively charged molecules and have a high affinity for the relatively negatively charged bacterial cells (Gilbert & Moore, 2005). This specific nature of QAC binding to bacterial cells offers bacteria a great potential for the development of resistance to QACs. Some bacteria are naturally resistant to certain compounds (Kumar & Schweizer, 2005; Langsrud *et al.,* 2003 a). One such method involves growth as a biofilm where cells generally survive mainly due to the inability of the disinfectants to reach the cells which results in the reduction of the susceptibility profile (Gilbert *et al.,* 1990; Brown & Gilbert, 1993, McDonnell & Russell, 1999; Campanac *et al.,* 2002). An example of this interaction is the virulent food-related staphylococci strains where biofilm formation was correlated to resistance to disinfectants (Moretro *et al.*, 2003). Another example of natural resistance to QACs is *Pseudomonas aeruginosa* cells that produce a lipopolysaccharide layer that prevents disinfectants from reaching the cells' outer membrane (Adair *et al.*, 1971; Méchin *et al.*, 1999).

Gram negative organisms are known to be more resistant to most antimicrobial agents (Kumar & Schweizer, 2005). This intrinsic resistance is attributed to the presence of enzymes that inactivate the drug or substrate, multidrug efflux pumps that pump these substrates out of the cell, changes in the fatty acid composition of the cell wall and growth in a biofilm where the substrate can simply not reach the bacterium, and lastly mutations that alter the target site (Putman *et al.,* 2000; Borges-Walmsley & Walmsley, 2001; Kumar & Schweizer, 2005). Resistance can also be acquired through the uptake of plasmids that carry resistance genes or through the horizontal transfer of resistance genes (Sidhu *et al.,* 2002).

The presence of efflux pumps are the most frequent strategy for the cell to reduce intracellular drug concentrations to sub-toxic levels, a very important mechanism of resistance in bacteria (Borges-Walmsley & Walmsley, 2001; Hegstad *et al.,* 2010). Efflux pumps are capable of removing QACs from the membrane core and effectively reducing the effectiveness of the QAC (Gilbert and Moore, 2005). These pumps include the ATP driven transporters and the proton pump antiporters. In Figure 1.3, a

schematic representation of the different efflux systems found in bacterial cells is displayed.

The proton pump antiporters contain one of three classes of antiporters; the major facilitator superfamily (MF), the small multidrug resistance family (SMR), the resistance nodulation division family (RND) while the ATP driven antiporters contains the ATP binding cassette (ABC) (Borges-Walmsley & Walmsley, 2001; Kumar & Schweizer, 2005; Putman *et al.,* 2000). Efflux systems are found in both gram negative and gram positive bacteria but efflux mediated resistance is more complex in gram negative bacteria because of their complex cell wall (Kumar & Schweizer, 2005; Sidhu *et al.,* 2002). The ABC transporters are rare in bacteria and are involved in uptake as well as efflux systems where energy for the transport is derived from the hydrolysis of ATP (Borges-Walmsley & Walmsley, 2001; Kumar & Schweizer, 2005; Putman *et al.,* 2000).

The proton pump antiporters function by transporting toxic compounds out of the cell via a transmembrane electrochemical gradient of protons or sodium ions, proton motive force and only differ in size (Putman *et al.,* 2000). The MF transporters are composed of about 400 amino acids that are arranged into 12 to 14 membrane spanning helices and have been found in both gram positive and gram negative bacteria (Borges-Walmsley & Walmsley, 2001; Kumar & Schweizer, 2005; Putman *et al.,* 2000). The *Staphylococcus* QacA and QacB proteins are part of this family of proteins (Gaze *et al.,* 2005; Kumar & Schweizer, 2005).

The RND transporters are composed of around 1000 amino acids and have a similar 12 helic structure as the MF (Kumar & Schweizer, 2005). They typically operate with a periplasmic membrane fusion protein and an outer membrane protein (Figure 1.3) allowing for transport of toxic compounds out through both the inner and the outer membrane of the gram negative bacteria (Putman *et al.,* 2000). QAC resistance genes in the *Staphylococcus* genus are widely spread amongst clinical isolates as well as isolates from the food industry (Bjorland *et al.,* 2005). The small multidrug resistance family includes; *smr, qacJ, qacH* and qacG and is found on small non-conjugated and large conjugated plasmids (Heir *et al.,* 1998; 1999; Bjorland *et al.,* 2001; 2003). This protein consists of 4 predicted transmembrane segments (Figure 1.4). These multidrug transport pumps do not have product specificity and thus could potentially mediate cross-resistance to a number of

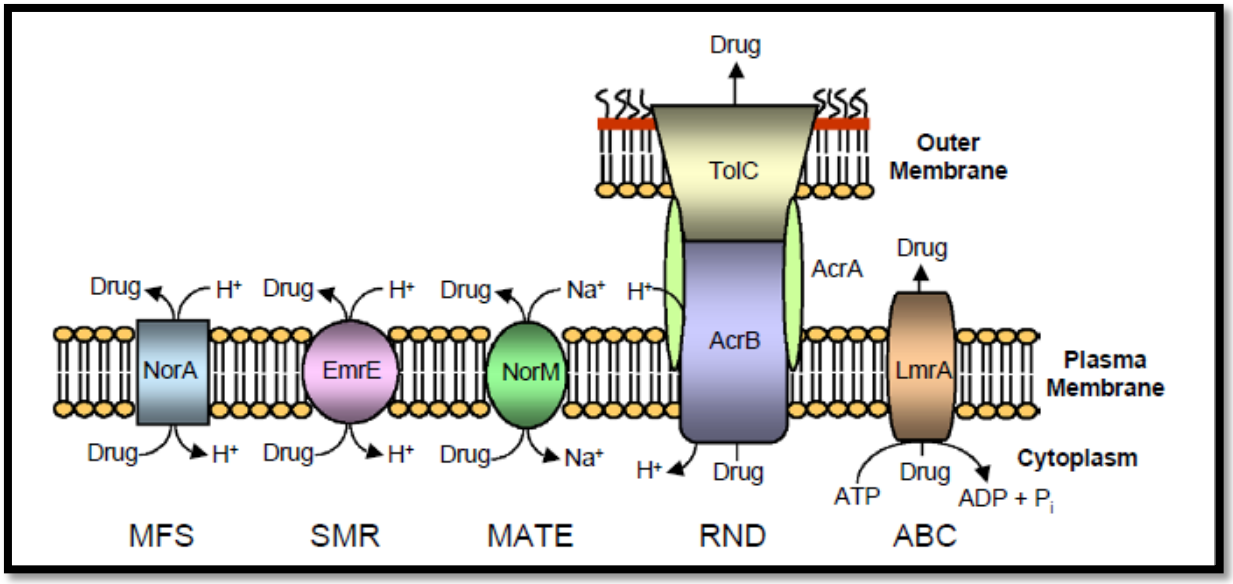

Figure 1.3. Schematic illustration of the main types of bacterial drug efflux pumps. Illustrated are *Staphylococcus aureus* NorA, a member of the major facilitator superfamily (MFS); *Escherichia coli* EmrE, a member of the small multidrug resistance (SMR) superfamily; *Vibrio parahaemolyticus* NorM, a member of the multidrug and toxic compound extrusion (MATE) superfamily; *E. coli* AcrB-TolC, a member of the resistance-nodulation-cell division (RND) superfamily; and *Lactococcus lactis* LmrA, a member of the ATP-binding cassette (ABC) superfamily. All pumps extrude the substrate chemically unaltered and in energy-dependent manner, using either an ion gradient (proton or Na^+) or ATP. Although the drug is in many instances pumped from the cytoplasm (as depicted here), there is increasing evidence that RND pumps can also acquire substrates either directly from the periplasm or from the outer leaflet of the cytoplasmic membrane (not shown for clarity). Whereas the cytoplasmic membrane is a phospholipid bilayer, the outer membrane consists of a phospholipid inner leaflet and a lipid A-containing outer leaflet. Lipid A is the membrane-anchoring domain of LPS, which, together with porins, gives the outer membrane its characteristic permeability properties (Kumar & Schweizer, 2005).

antimicrobial agents (Borges- Walmsley & Walmsley, 2010). Several multidrug transporters can be present within the same bacterium and the availability of these different transporters may contribute to bacterial resistance against a wide range of substrates (Putman *et al.,* 2000). QACs have been used in the industry for a very long time with no seeming reduction in their effectiveness, but various reports have indicated resistance against QAC in the food and medical industry (Gilbert & Moore, 2005). Most of the resistance reported refers to changes in the MIC and does not affect the efficacy of the QAC which is generally higher than the MIC (Gilbert & Moore, 2005). Changes in the MIC level have been shown to be as a result of changes in the phospholipid content of the membrane or the presence of multi-drug efflux pumps (Wright & Gilbert, 1987; Heir *et al.,* 1998; Gilbert & Moore, 2005).

During multiple sequence alignments, a number of conserved amino acid sequence motifs throughout the multidrug transport families have been identified (Pulsen & Skurry, 1993; Paulsen, 1996 a; b; Rouch *et al.,* 1990; Saier *et al.,* 1994). A structural model of the SMR protein is presented in Figure 1.4. The presence of the charged amino acid, glutamic acid suggests that these motifs play an important role in the binding and transport of charged compounds (Putman *et al.,* 2000). During the binding of toxins onto the glutamic residues, they deprotonate and undergo a conformational change, the binding site closes and opens on the other face of the membrane and subsequently the release of the molecules (Muth & Schuldiner, 2000). These conserved sequences could be used to identify possible new proteins belonging to the SMR families (Putman *et al.,* 2000).

Table 1.1. Number of sequenced and functionally characterized multidrug transporters of various families in selected bacteria (Putman *et al.,* 2000).

Organism	MFS		SMR	RND	MATE
	12-TMS cluster	14-TMS cluster			
Bacillus subtilis	2	1	2	0	0
Escherichia coli	3	1	1	3	1
Staphylococcus aureus	1	2	4	0	0
Mycobacterium tuberculosis	1	1	1	0	0
Pseudomonas aeruginosa	0	0	0	4	0

1.2.2. Link between disinfectant resistance and antibiotic resistance

Disinfectants have a longer history in the clinical environment than antibiotics in the fight against bacterial infections (Hegstad *et al.,* 2010). Bacterial resistance to antibiotics has been a great concern ever since their inception and a big concern is the possible link between antibiotic and disinfectant resistance because genes conferring resistance to both can sometimes be found on the same plasmid (Sidhu *et al.,* 2001). QAC resistance genes have been shown to be found on class 1

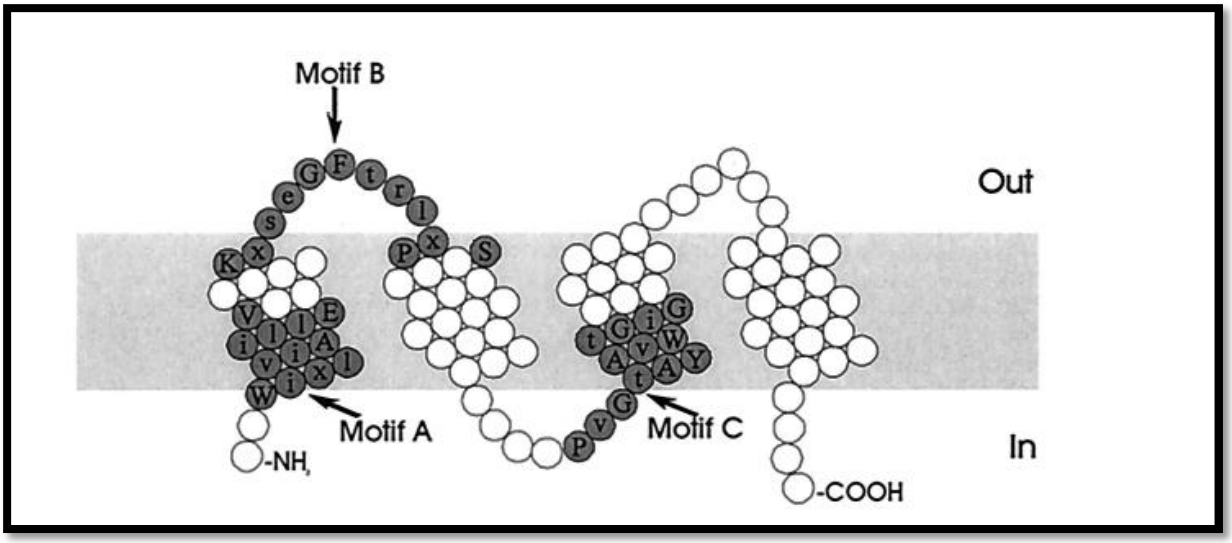

Figure 1.4. Structural model for multidrug transporters of the SMR Family. The residues constituting the conserved sequence motifs are shaded. (Putman *et al,* 2000). The consensus sequences of the motifs are displayed as follows: x, any amino acid; capital letters, amino acid occurs in >70% of the examined sequences; lowercase letter, amino acid occurs in >40%; (x), amino acid not always present (Paulsen *et al,* 1995; 1996 a; b).

integrons isolated from clinical environments, but they have also been found in environmental bacteria as part of an antibiotic resistance cassette (Gillings *et al.,* 2008 a; b; 2009 a; b; Hegstad *et al.,* 2010). Integrons are recombination and expression systems where genes are captured as part of genetic elements known as gene cassettes (Hardwick *et al.,* 2008; Recchia & Hall, 1995). QAC and antibiotic resistance genes are usually carried by cassettes (Gaze *et al.,* 2005).

The co-resistance of QAC and antibiotics could be achieved by linkage of different resistance mechanisms on the same plasmid, transposon or integron or any combination of these (Hegstad *et al.,* 2010). The localization of these QAC determinants on different mobile elements, may contribute to the transfer of resistance to other bacteria. Gene cassettes might be readily shared between different integron classes found in environmental, commensal and pathogenic bacteria suggesting that class 1 integrons in pathogens have access to a cast pool of gene cassettes any of which could confer a phenotype of clinical relevance (Gillings *et al.,* 2009 a; b).

1.3. NanoSAM

Yoshimada and Hiyama (2007) reported on the morphological changes induced by didecyldimethylammonium chloride (DDAC) in *Escherichia coli* cells. Similar changes were observed in *Lawsonia intracellularis* cells treated with QACs (Wattanaphansak *et al.,* 2010). Scanning electron microscopy (SEM) and NanoSAM can be used to determine the morphological changes caused as a result of QAC treatment. SEM is a powerful tool that can resolve the structure of subcellular compartments and could be a useful tool to record the morphological changes associated with antibiotic activity (Koster and Klumperman, 2003). Scanning Auger microprobes in scanning auger spectroscopy (AES) are used as surface analytical instruments operating under ultrahigh vacuum conditions with secondary electron detectors added for SEM imaging (Hochella *et al.,* 1986). It allows for semi-quantitative elemental analysis on samples several orders of magnitude smaller than those analysed using SEM (Hochella *et al.,* 1986; Swart *et al.,* 2010).

Applications of AES/SAM generally involve the near-surface analysis of conductors and semiconductors (Calvo-Bario *et al.,* 2001; Hochella *et al.,* 1986). When an electron beam is used on a specimen it produces secondary, backscattered and Auger electrons which are then subsequently collected by various detectors in the specimen chamber and used to form an image (Vernon-Parry, 2000). An auger electron is produced when an atom undergoes inner-shell ionization by electron bombardment and is released (Hochella *et al.,* 1986; Vernon-Parry, 2000). The electron beam in AES is used to excite Auger electrons from a solid (Hochella *et al.* 1986). The inner-shell vacancy are subsequently filled by an electron from a higher energy level, and the energy released in this de-excitation process results in the ejection of a third electron, the auger electron (Figure 1.5; Hochella *et al.,* 1986). All elements have a unique set of electron-binding energies; all elements detectable by AES have a unique Auger spectrum (Hochella *et al.,* 1986). Nano Scanning Auger Microscopy (NanoSAM) brings a new and powerful means to view and analyse the composition in microorganisms as the SEM mode allows for bigger magnification and resolution of the samples (Swart *et al.,* 2010). SAM has also been used to perform in depth studies where the argon ion gun has been used for targeted etching on materials such as semi-conductors (Calvo-Bario *et al.,* 2001), and more recently biological material during the study of the sexual structures of yeasts (Swart *et al.,* 2010). The argon gun was used to etch through the sample in nanometer thick

segments while the SAM and SEM modes were applied to analyse and visualise the elemental composition and 3D ultrastructure of ascospore structures.

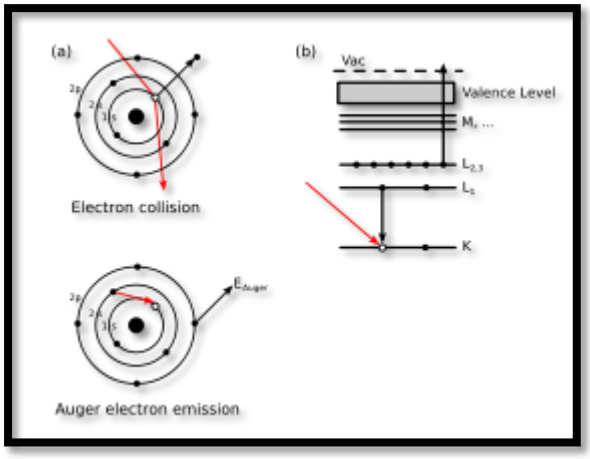

Figure 1.5. Two views of the Auger process. (a) Sequential steps involved in Auger de-excitation. An incident electron (or photon) creates a core hole in the 1s level. An electron from the 2s level fills in the 1s hole and the transition energy is imparted to a 2p electron which is emitted. The final atomic state thus has two holes, one in the 2s orbital and the other in the 2p orbital. (b) Auger de-excitation using spectroscopic notation, KL_1L_2. (Hochella *et al.,* 1986; Vernon-Parry, 2000; Calvo-Barrio *et al.,* 2001)

1.4. Real time PCR

Real time PCR has become a valuable technique that can be used in the identification of bacteria containing *qac* resistance genes that are potentially in low copy numbers within the bacterium. This technique has become the preferred method for expression studies because of its sensitivity and efficiency (Pfaffl *et al*., 2002). It has been utilised in the search for *qac* gene cassettes in the natural environment where Gillings and co-working (2008 b) showed that *qac* gene cassettes are widespread in the natural environment residing on class 1 integrons previously thought to be present only in clinical environments. This technique could be employed in the gene expression studies where the expression of the *qac* genes could be monitored.

Real-time PCR is a PCR technique where amplification and detection of product is combined into a single step where specific fluorescence signals are measured with each step (Ririe *et al.,* 1997; Stöcher *et al.,* 2002; Wong & Medrano, 2005; Schefe *et al.,* 2006). This is achieved using a variety of different fluorescent dyes that

intercalate onto double stranded DNA thus resulting in an increase in fluorescence intensity as product concentration increases (Higuchi *et al.,* 1993).

PCR kinetics can be divided into four major phases namely the linear lag phase, early exponential phase, exponential phase and plateau phase (Figure 1.6). The linear lag phase of PCR is during the beginning stage of PCR where there is very little product and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time (Wong & Medrano, 2005). At the early exponential phase the amount of fluorescence reaches a threshold where it is significantly higher than background levels and the cycle at which this occurs is known as cycle threshold (C_t) or crossing point (CT) and this is visualised in a half logarithmic plot. This CT value is used as a representative of the starting copy number of the original template copy number and is used to calculate experimental results (Heid *et al.,* 1996).

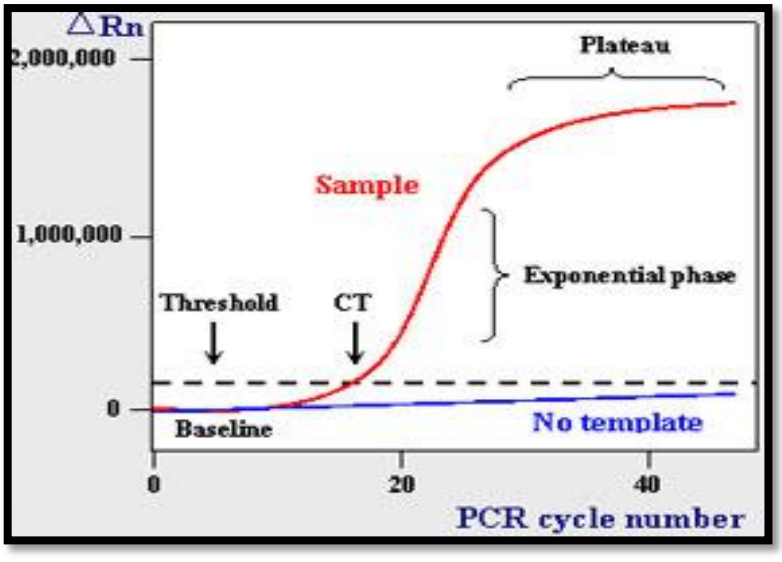

Figure 1.6. Major phases of PCR. CT is the threshold cycle where fluorescence increases above the threshold fluorescence indicating the amplification of the sample (Roche Molecular Biochemicals Technical Note No. LC 6/99).

The greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower CT. PCR reaches its optimal amplification period during the log linear phase where in ideal reaction conditions the PCR product doubles after every cycle. Finally, the plateau is reached

when reaction components become limited and the fluorescence intensity is no longer useful for data calculation (Wong & Medrano, 2005). The benefits to using real time PCR include the following: it can produce quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude and does not require post-amplification manipulation; Real time assays are 10 000 – 100 000-fold more sensitive than RNase protection assays; 1000-fold more sensitive than dot blot hybridization, and can even detect a single copy of a specific transcript (Wong & Medrano, 2005). The fluorescent dye used in real time PCR, SYBR green, pose certain disadvantages as it detects all double stranded DNA including primer dimers and other undesired products and could lead to the generation of false positives (Wong & Medrano, 2005). The use of sequence specific probes, such as hybridization probes and hydrolysis probes increase the specificity thus the increase of fluorescence is a more accurate measure for the increase in product (Zimmermann & Mannhalter, 1996; Wong & Medrano, 2005).

To compensate for the possibility of obtaining undesired products during SYBR green amplification, melting curve analysis can be performed as a control measure. Melting curve analysis relies on the GC content, length and the sequence of PCR products and is performed during the PCR process by monitoring the fluorescence change of product as the temperature of reaction is raised to the denaturation temperature (Ririe *et al.*, 1997). With this technique products can be differentiated based on their GC/AT ratio and because the melting curve of a product is dependent on GC content, length and sequence, PCR products can be distinguished by their unique melting curves (Ririe *et al.*, 1997; Wong & Medrano, 2005).

Empirical formulas predict that a 0% GC duplex would melt 41°C lower than a 100% GC duplex (Ririe *et al.,* 1997). Given the same GC content, a 40-base-pair primer dimer should melt 12°C below a 1000-bp product (Ririe *et al.,* 1997). Because of length differences, nonspecific products usually melt at a lower temperature than desired PCR products. Melting curve analysis is frequently used in single nucleotide polymorphism analysis and mutation and genotype determinations (Roche Molecular Biochemicals Technical Note No. LC 6/99).

1.4.1. Quantitative Real Time PCR

Quantitative real time PCR (qPCR) is the measurement of the amount of amplified product in the exponential phase by reference to the dilution series of a standard (Zimmermann & Mannhalter, 1996). There is currently no data available on the

expression of the individual *qac* genes in bacterial strains. During relative quantification, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator. When using a calibrator, the results are expressed as a target/reference ratio.

There are numerous mathematical models available to calculate the mean normalized gene expression from relative quantification assays. Amplification efficiency of the reaction is an important consideration when performing relative quantification. Traditionally, the amplification efficiency (E) of a reaction is calculated using data collection from a standard curve using the following formula: $E = 10^{-(\frac{1}{slope})}$. During an ideal PCR reaction amplification would be achieved where doubling of the gene-specific product occurs after each amplification cycle and would result in an efficiency of 100% or 1 (Schefe *et al.,* 2006). The PCR efficiency E, is defined by the CT value and the resulting gene expression ratios (Schefe *et al.,* 2006; Peirson *et al.,* 2003). During the exponential phase, the absolute fluorescence increase at each PCR cycle for each individual sample reflects the true reaction kinetics of that sample. Consequently, data collected during the exponential phase can be log-transformed and plotted with the slope of the regression line representing the sample's amplification efficiency (Figure 1.7).

Quantification of the PCR product can be achieved with the standard curve method or the comparative CT method. When using the standard curve method, the quantity of each experimental sample is first determined using a standard curve and then expressed relative to a single calibrator sample. The calibrator is designated as one-fold, with all experimentally derived quantities reported as an n-fold difference relative to the calibrator. Because sample quantity is divided by calibrator quantity, standard curve units are eliminated, requiring only the relative dilution factors of the standards for quantification. This method is often applied when the amplification efficiencies of the reference and target genes are unequal. It is also the simplest method of quantification because it requires no preparation of exogenous standards, no quantification of calibrator samples, and is not based on complex mathematics. This method does not incorporate an endogenous control or the use of multiple housekeeping genes and results must still be normalized.

The comparative CT ($2^{-\Delta\Delta C_P}$) method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample (Livak & Schmittgen, 2001; Pfaffl, 2001). The Pfaffl model combines gene quantification and normalization into a single calculation. This model

incorporates the amplification efficiencies of the target and reference (normalization) genes to correct for differences between the two assays. Relative expression ratios are defined by the expression of a gene of interest (GOI) in one specific sample compared to a reference sample (Schefe *et al.,* 2006). Normalization of gene expression data is used to correct sample-to-sample variation (Theis *et al.,* 2007). Starting material obtained from different individuals usually varies in tissue mass or

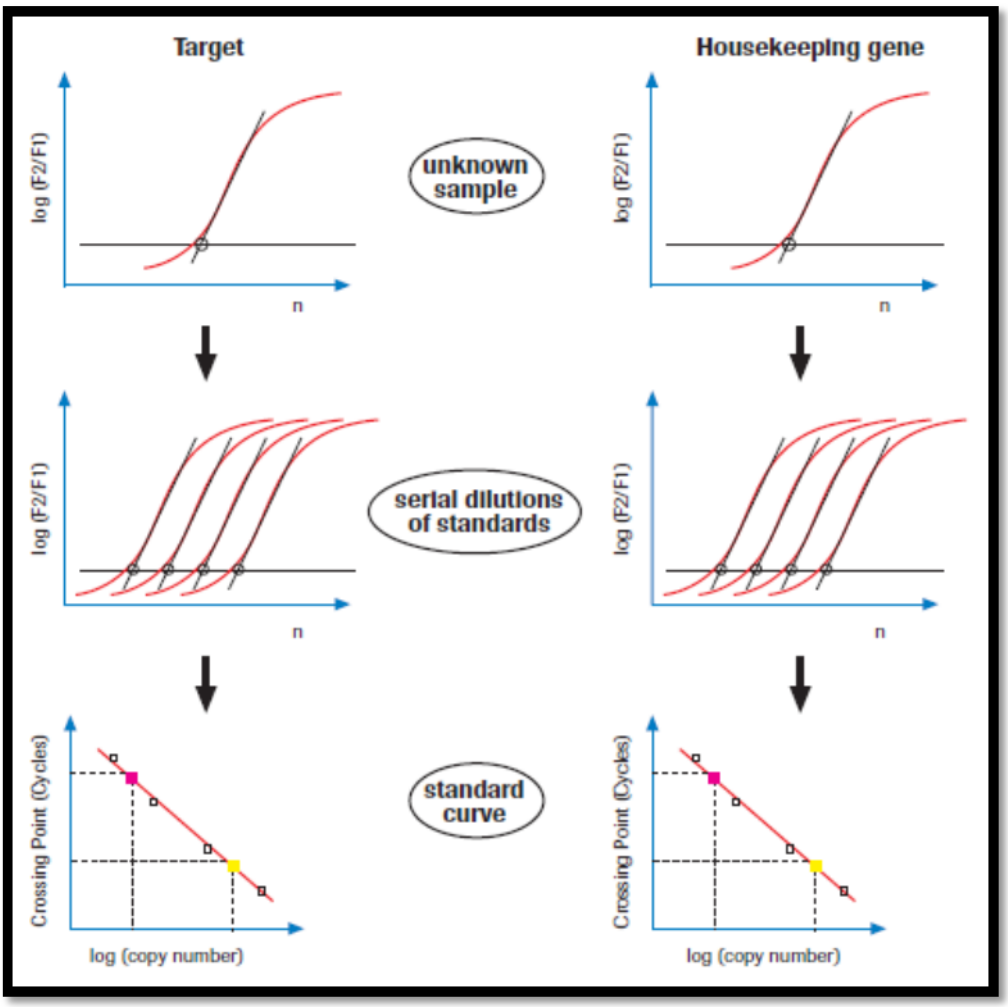

Figure 1.7. General quantification concept for the determination of the concentration of a target and housekeeping gene using separate external standards, one for the target and one for the housekeeping gene. (Roche Molecular Biochemicals Technical Note No. LC 6/99)

cell number, RNA integrity or quantity or experimental treatment. Real-time PCR results are usually normalized against a control gene (calibrator) that may also serve as a positive control for the reaction (Wong & Medrano, 2005). The ideal control gene

should be expressed in an unchanging fashion regardless of experimental conditions (Theis *et al.*, 2007). In terms of normalization, the use of multiple housekeeping (HK) genes is the most accurate method (Wong & Medrano, 2005).

Traditionally, genes thought to have stable expression have been employed as controls in gene expression assays, but normalization with a single HK gene can falsely bias results. (Theis *et al.*, 2007). It is very important to validate its stability with every sample extracted from different cultivation conditions rather than relying on previously published materials. In theory PCR is quite robust and predictable, but in actuality, minor variations in reaction components, thermal cycling conditions, and miss-priming events during the early stages of the reaction can lead to large changes in the overall amount of amplified product.

Recently it was shown that the QAC resistance genes can be found on class 1 integrons that are widely spread in the natural environment and not only in a clinical environment (Gaze *et al.*, 2005; Gillings *et al.*, 2008 a; b ; 2009 a; b). Smith and co-workers (2008) showed that the expression of the *qac* genes increased in the presence of disinfectants when assayed with a luciferase reporter. They focused on the expression of the *qac* resistance genes by looking at the expression of the *qacR* gene that regulates the expression of the *qac* genes *qacA* and *qacB* genes. Gene expression requires sensitive and reproducible measurements for specific mRNA sequences (Bustin, 2000). Real time PCR is one of the best methods to determine gene expression as it is a very sensitive method for the detection of low abundance mRNA (Bustin, 2000; Nicot *et al.*, 2005).

1.5. Introduction to study

Quaternary ammonium compounds (QAC) have been widely used in the medical and food industries to control pathogenic bacterial growth. These compounds have since been used in a wide variety of pharmaceutical products as well. The over use of these products could put a selective pressure on bacteria causing them to become less sensitive to these compounds.

The existence of bacteria resistant to QACs is of great concern especially in this time when the poultry industry is headed towards a post antibiotic era. The presence of *qac* genes have been identified in clinical environments because this is where QACs are used on routine basis. Recently the *qac* resistance genes were isolated in food industry and in veterinary environments.

After the ban on the use of antibiotics as growth promoters, the poultry industry in Europe suffered financial losses due to bacterial infections. The use of antibiotics as growth promoters also helped in the protection of the animals against bacterial infections and soon afterwards antibiotics were used to control bacterial infections. Restrictions are in place to limit the use of antibiotics, used for human health, in animals. The veterinary industry is headed for a looming post-antibiotic era. Bragg and Plumstead (2003) have shown that a continuous disinfection program showed promising results in the control of bacterial infections. In order not to end up with the same problems as antibiotics with disinfectants, the existence of resistance to QAC needs to be investigated.

1.6. Aims of the study

The aim of this study was to determine whether there are potential problems with resistance to QACs and whether the MIC to QACs were related to the presence or absence of the QAC resistance genes. There is much speculation as to the mode of action of QACs and their effect on the bacterial cell. During this study the mode of action of QAC was studied and a new and exciting technique (NanoSAM) was used to determine the morphological changes in cells as a result of treatment with QACs.

Real time PCR was also used to look at the difference in the expression of the QAC resistance genes in the presence of different QAC concentrations. The QAC resistance genes are generally carried on plasmids and introns and it was found to be widely distributed in bacteria. The presence of these genes in different bacterial cultures was investigated and related back to the selective pressure placed on bacteria as a result of the overuse and to an extent the abuse of these compounds.

Chapter 2
Evaluation of Quaternary Ammonium Compound resistance in bacteria through the detection of QAC resistance genes and the determination of minimum inhibitory concentrations.

2.1. Introduction

Alternatives to the use of antibiotics in the control of bacterial infections have been investigated ever since the restrictions placed on the use of antibiotics in the animal health (Joerger, 2003; Nelson *et al.,* 2007). The use of disinfectants and in particular, QAC based disinfectants could possibly be the agricultural industries' last resort in the fight against bacterial infections in animals. Acquiring genes such as antibiotic resistance, heavy metal resistance or QAC resistance genes has enabled bacteria to survive harsh environments (White & McDermott, 2001). These resistance genes are usually acquired via plasmids, transposons or integrons (White & McDermott, 2001; Russel, 2002; Chapman, 2003; Langsrud *et al.,* 2003 b).

QAC efflux via the small multidrug resistance (SMR) family proton driven antiporters are the most important method of bacterial resistance against QACs. The genes that code for SMR, *qacG, qacJ, qacH* and *smr,* have been identified in food-borne, clinical and veterinary isolates (Smith *et al.,* 2008). These small proteins are of great interest due to their lack of substrate specificity and their ability to confer increased tolerance to QACs in bacteria (Bjorland *et al.,* 2003). These QAC resistance genes have been identified on different plasmids and the sequences are available on NCBI database.

The basic use of disinfectants is for the disinfection of surfaces and they are generally used at concentrations noticeably higher than their minimal inhibitory concentrations (MICs) (White & McDermott, 2001), but practical experience has indicated that many products in the market have application rates which are close to the MIC (Bragg, 2012). The possibility still remains though, that some bacteria are exposed to low concentrations that could allow for the survival, and thus the development of resistance against antibacterial agents (Sidhu *et al.,* 2001; White & McDermott, 2001; Russel, 2002; Chapman, 2003; Langsrud *et al.,* 2003 b).

In the current study the presence of *qac* resistance genes were investigated and the minimal inhibitory concentration (MIC) was determined in order to relate resistance against selected QACs, to the presence of the different *qac* resistance genes.

2.2. Materials and Methods

2.2.1. Bacterial strains

Bacterial strains harbouring one of each of the Quaternary ammonium compound (*qac*) resistance genes were obtained from Prof Bjorland, Department of production animal clinical sciences, Norwegian School of Veterinary Science, Oslo Norway. These strains were subsequently named according to the possible gene they harbour as well as a veterinary biotechnology (VB) number (Table 2.1). *Staphylococcus aureus* strain ATCC 25923 was obtained from the University of the Free State's bacterial culture collection. A strain of avian pathogenic *Escherichia coli* was obtained from a previous study in the veterinary biotechnology laboratory (Van der Westhuizen, 2010). Bacterial strains were obtained in freeze dried form and were reconstituted in tryptic soy broth (TSB) for 24 h at 37°C. Bacterial strains were routinely cultivated in TSB at 37°C for 18 h.

The bacterial strains were stored in microbanks (Prolab) where beads suspended in broth, was inoculated with a colony, resuspended and the broth completely aspirated and the microbank stored at -20°C. The bacterial cells adhere to the bead and can be reconstituted by inoculating a bead into growth medium or streaking on an agar plate. One to three beads were either streaked on Tryptic soy agar (TSA) plates or suspended in TSB and incubated at 37°C for 18 h. One microbank was used as a master culture and 3 working microbanks were made from the master and used in routine experiments to ensure the strains stayed the same. Every 3 months the culture was streaked on TSA plates to ensure that no contamination was present.

2.2.2. Identification of strains

2.2.2.1. Biochemical method

Bacterial strains (Table 2.1) were identified by gram stain as well as growth on the selective agar. Bacteria were plated out on TSA plates for single colonies and incubated overnight at 37°C and a gram strain performed on fresh single colonies. Gram positive cocci were streaked on mannitol salt agar (Merck, South Africa) as well as Baird parker agar (Merck, South Africa) and incubated overnight at 37°C.

Bacterial strains were plated out on Mueller-Hinton agar (Merck, South Africa) and tested for their sensitivity to flurazolidone (Rosco Diagnostics) by placing a disk containing the antibiotic on the culture and determining their sensitivity pattern as only bacterial strains sensitive to flurazolidone were allowed to be tested using the Staph-zym™ kit.

Table 2.1. Bacterial strains used during this study, strain description and the source of isolation

Strain	Strain description	Source
VB1*_qacG	Resistant (*qacG*)	Norwegian School of Veterinary Science, Oslo Norway
VB2_qacH	Resistant (*qacH*)	Norwegian School of Veterinary Science, Oslo Norway
VB3_qacJ	Resistant (*qacJ*)	Norwegian School of Veterinary Science, Oslo Norway
VB4_smr	Resistant (*smr*)	Norwegian School of Veterinary Science, Oslo Norway
VB5_qacA	Resistant (qacA)	Norwegian School of Veterinary Science, Oslo Norway
VB6	Gram positive cocci	University of the Free State culture collection
Staphylococcus aureus ATCC 25923	Susceptible strain	University of the Free State culture collection
Avian Pathogenic *Escherichia coli* (APEC)	Gram negative rod	Poultry pens

*VB – veterinary biotechnology number

Gram positive bacterial strains were further identified with the Staph-zym™ kit (Rosco Diagnostics) according to the manufacturer's protocol. The Staph-zym™ system is a combination of enzymatic and antimicrobial identification. Briefly, test strains were cultivated on Mueller-Hinton agar plates and colonies selected and

suspended in 3 ml physiological saline (0.9% NaCl) to a turbidity equal to the McFarland No 2 standard (OD of approx. 0.6; 600nm). The bacterial suspension (250 µl) was inoculated in each of 10 trays containing different tablets for detecting different enzymatic activity and incubated at 37°C overnight and the results that appeared as a specific colour reaction was recorded. The test strains sensitivity to deferoxamine, novobiocin and polymyxins were recorded according to the natural susceptibility patterns on Mueller-Hinton agar. All bacterial cultures used during the study are shown in Table 2.1.

2.2.2.2. Identification of strains using the 16S rRNA gene amplification

DNA was extracted using an alkaline lysis method as described by Labuschagne & Albertyn (2007). *S. aureus* was cultivated in tryptic soy broth overnight. Cells were harvested and lysed by resuspending cells in 500 µl cell lysis buffer (100 mM tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 1% SDS) and lysis was aided by adding 200 µl glass beads (Sigma-Aldrich, South Africa). Thereafter the samples were vortexed for 4 minutes (min), cooling on ice for 4 min and 275 µl ammonium acetate (7 M, pH 7.0) was added to the samples. After incubation at 65°C for 5 min and cooling on ice for 5 min, 500 µl chloroform was added to aid in the denaturation of cell proteins. Thereafter the samples were vortexed and centrifuged (20 000 x *g*, 5 min at 4°C). The supernatant containing DNA was transferred to a clean tube and DNA was precipitated with 700 µl isopropanol and centrifuged (20 000 x *g*, 5 min at 4°C). The pellet was washed with 70% ethanol, dried and re-dissolved in 100µl nuclease free water.

The 16S rRNA gene was amplified using universal 16S rRNA primers 8F and 1525R (Table 2.2). PCR was carried out in a total reaction volume of 50 µl consisting of 5 µl of DNA template, 1 µl of 10 mM dNTP mix, 0.5 µl of each 100 mM primer, 5 µl of $10\times$ ThermoPol Reaction Buffer and 1 U of *Taq* DNA Polymerase (New England Biolabs Inc.) made up to 50 µl with sterile Milli-Q water. Reactions were thermocycled on a 2720 Thermal cycler (Applied Biosystems) starting with an initial denaturation step at 94°C for 2 min. Twenty five cycles of denaturation at 94°C for 30 seconds (sec), annealing at 53°C for 30 sec and elongation at 72°C for 30 sec were carried out followed by a final elongation step at 72°C for 7 min. Amplified fragments were observed under ultraviolet (UV) illumination on a 1% agarose gel stained with gold view (Peqlabs).

2.2.3. Amplification of the Quaternary ammonium resistance genes

Colony PCR was performed on the bacteria listed in Table 2.1 to detect the quaternary ammonium (*qac)* resistance genes, *smr, qacJ, qacG* and *qacH*. The PCR were performed using the primers listed in Table 2.2. Colony PCR was carried out by selecting a colony, re-suspending it in nuclease free water and incubating it at 95°C for 15 min and cooling on ice. The suspension was briefly centrifuged to pellet the cell debris.

Five micro litres of the supernatant was used as template in a PCR reaction volume of 50 µl consisting of 0.2 mM dNTP mix, 1 mM primer, 1X ThermoPol Reaction Buffer and 1 U of *Taq* DNA Polymerase (New England Biolabs Inc.) made up to 50 µl with nuclease free water. Reactions were thermocycled on a 2720 Thermal Cycler (Applied Biosystems) starting with an initial denaturation step at 94°C for 1 min. Thirty five cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec and elongation at 72°C for 60 sec were carried out followed by a final elongation step at 72°C for 5 min.

Amplified fragments were observed under ultraviolet (UV) illumination on a 1% agarose gel stained with gold view (Peglabs).DNA was purified from solution using the Illustria™ DNA and Gel Band Purification Kit (GE Healthcare) following the manufacturer's instructions. A GFX column was placed in a collection tube per purification to be performed from solution and 500 µl of capture buffer added to the column to ensure binding of the PCR product to the spin column. The PCR DNA solution was transferred to the column and mixed thoroughly by pipetting up and down 6 times. The column was centrifuged (Eppendorf Centrifuge 5417R) at full speed for 30 sec and the flow through discarded.

The column was placed back into the collection tube, 500 µl of wash buffer added to remove excess dNTPs, enzymes and primers not used during amplification and the column centrifuged at full speed for 30 sec. The collection tube was discarded, the column transferred to a sterile microcentrifuge tube and 50 µl of elution buffer (10 mM Tris-HCl, pH 8.0) added directly to the top of the glass fibre matrix of the column. The column was incubated at room temperature (RT) for 1 min and centrifuged at full speed for 1 min to recover the purified DNA. The concentration of DNA was measured with a nanodrop.

2.2.4. DNA sequencing reactions

DNA Sequencing reaction was performed using the BigDye terminator v. 31 kit (Applied Biosystems). The sequencing PCR was carried out in a total reaction volume of 10 µl or 40 µl for sequencing of *smr* genes and 16S rRNA genes respectively. The sequencing reaction consisted of 10 ng PCR product, 0.5 µl of

Table 2.2. Sequence information on 16S rRNA universal primers and small multidrug resistance primers used in this study

Gene amplified	Primer name	Primer sequence	Tm
16s rRNA	8F	5'-AGAGTTTGATCNTGGCTCAG-3'	50.5°C
16s rRNA	1525R	5'-AAGGAGGTGWTCCARCC-3'	48.7°C
qacG	qacG rev	5'-CTCAATTGCAACAGAAATAATCG-3'	50.7°C
qacG	qacG forw	5'-GGCTTTCACCAAATACATTTAAG-3'	50.5°C
qacH	qacH rev	5'-GTGTGATGATCCGAATGTGTT-3'	53.1°C
qacH	qacH forw	5'-CAGTGAAGTAATAGGCAGTGC-3'	53.4°C
qacJ	qacJ rev	5'-CGTTAAGAAGCACAACACC-3'	51.6°C
qacJ	qacJ forw	5'-GCGATTATAACTGAAATAATAGG-3'	47.1°C
Smr	qacC rev	5'-AACGAAACTCAGCCGACTATG-3'	54.9°C
Smr	qacC forw	5'-AAACAATGCAACACCTACCAC-3'	55.1°C

reaction premix, 1 µl sequencing primer (3.2 mM), 2 µl dilution buffer and nuclease free water to a final volume of 10 µl. In the case of the 16S rRNA sequencing the reaction consisted of 40 ng PCR product, 4 µl of premix, 1 µl sequencing primer (6 mM), 6 µl dilution buffer and nuclease free water to a final volume of 40 µl. Reactions were thermocycled starting with an initial denaturation step at 96^{o}C for 1 min. Twenty five cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and elongation at 60°C for 4 min were carried.

The sequencing reaction was cleaned using an EDTA/ethanol precipitation method. The reaction volume was adjusted to 20 µl and transferred to a centrifuge tube containing 5 µl EDTA (125 mM) and 60 µl absolute ethanol, vortexed for 5 sec and

allowed to precipitate at room temperature for 15 min. The mixture was centrifuged for 10 min (20 000 x *g,* 4°C) and the supernatant completely aspirated and the pellet washed with 200 µl 70% ethanol. The pellet was dried and sent for sequencing at the University of the Free State biosequence lab. Sequencing data was analysed with Geneious$^{(R)}$ software.

2.2.5. Multiplex PCR

Colony PCR was performed on the small multidrug resistance genes (SMR), *smr, qacJ, qacG* and *qacH* genes and amplified using the primers listed in Table 2.2. Colony PCR was carried out by selecting a colony, re-suspending it in nuclease free water and incubating it at 95°C for 15 min and cooling on ice. The suspension was briefly centrifuged to pellet the cell debris. Ten micro litres of the supernatant was used as template in a PCR reaction volume of 50 µl consisting of 1 µl of 10 mM dNTP mix, 1 µl of each 20 mM primers (*qacG*, *qacH*, *qacJ* and *qacC*), 5 µl of $10 \times$ ThermoPol Reaction Buffer and 1 U of *Taq* DNA Polymerase (New England Biolabs Inc.) made up to 50 µl with nuclease free water. Reactions were thermocycled starting with an initial denaturation step at 94°C for 1 min. Thirty five cycles of denaturation at 94°C for 30 sec, annealing at 45°C for 30 sec and elongation at 72°C for 60 sec were carried out followed by a final elongation step at 72°C for 5 min. Amplified fragments were observed under ultraviolet (UV) illumination on a 1% agarose gel stained with gold view (Peglabs).

2.2.6. Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) of bacterial strains to the QACs didecyldimethylammonium Chloride (DDAC) (Uniquat 2280, Lonza, USA), benzalkonium chloride (BC) (Merck, South Africa), alkylbenzyldimethylammonium chloride (AAC) (Merck, South Africa) and Virukill (ICA chemicals international) was determined using a microtiter assay. Serial dilutions at 2 µg ml^{-1} intervals of the QACs starting with a concentration of 187.5 µg ml^{-1} were made in 100 µl Mueller Hinton broth (Merck, South Africa) and 2×10^8 CFU ml^{-1} cells were inoculated and incubated at 37°C for 16 h. The MIC was the concentration at which no growth was observed after this extended contact time. All MIC were performed in triplicate

MICs against the above mentioned QACs were determined with a contact time of 20 min. Bacterial strains were cultivated in TSA for 4 h and transferred to the QACs with a starting concentration of 100 g l^{-1} for BC and AAC and 1.56 g l^{-1} for DDAC and

Virukill, and serially diluted at 2 µg ml^{-1} intervals in Mueller Hinton Broth. The bacterial culture was inoculated at a concentration of 2×10^8 CFU ml^{-1} in the different QACs and after a contact time of 20 min at 37°C, 20 µl of the culture was inoculated in 200 µl Mueller Hinton Broth and incubated overnight at 37°C The lowest concentration were no growth was recorded was the MIC. All MIC determinations were performed in triplicate.

2.3. Results

2.3.1. Identification of bacterial strains

Bacterial strains were initially identified using standard microbiological techniques. Gram staining revealed gram positive cocci and thus the strains were further identified using selective media, mannitol salt agar as well as Baird parker agar. The bacterial strains, VB1_qacG, VB2_qacH, VB3_qacJ and VB4_smr, VB5_qacA, VB6 and ATCC displayed the typical black colonies of *Staphylococcus* species on Baird Parker agar. During growth on mannitol salt agar the strains VB1_qacG, VB2_qacH, VB3_qacJ and VB4_smr, VB5_qacA, and ATCC caused the agar to change the phenol red indicator from red to yellow indicating mannitol fermentation. The strain VB6 did not cause any change in colour, but did grow on mannitol salt agar. The strains were also differentiated from *Micrococcus* by testing their sensitivity to furazolidone, *Micrococcus* is resistant to flurazolidone and Staphylococci are sensitive to it. All strains tested were sensitive to flurazolidone.

Bacterial strains obtained were suspected to be *Staphylococcus aureus* and were subsequently identified with the aid of the Staph-zym kit. The kit identifies gram positive bacteria using enzymatic tests where results are indicated as a specific colour change based on the organism's ability to utilize substrates (Figure 2.1) as well as their susceptibility to Deferoxamine, Novobiocin and Polymyxin. Results were tabulated on a record slip where a specific number was assigned for every positive test and zero for every negative. A five digit number (Table 2.3) was then generated that was compared to the staph-zym database for *Staphylococcus* species to identify the bacteria. The QAC resistant strains, VB1_qacG, VB2_qacH, VB3_qacJ and VB4_smr, VB5_qacA as well as the QAC susceptible strain ATCC 25923 was identified as *S. aureus* (Table 2.4). The strain VB6 was identified as *Staphylococcus intermedius.*

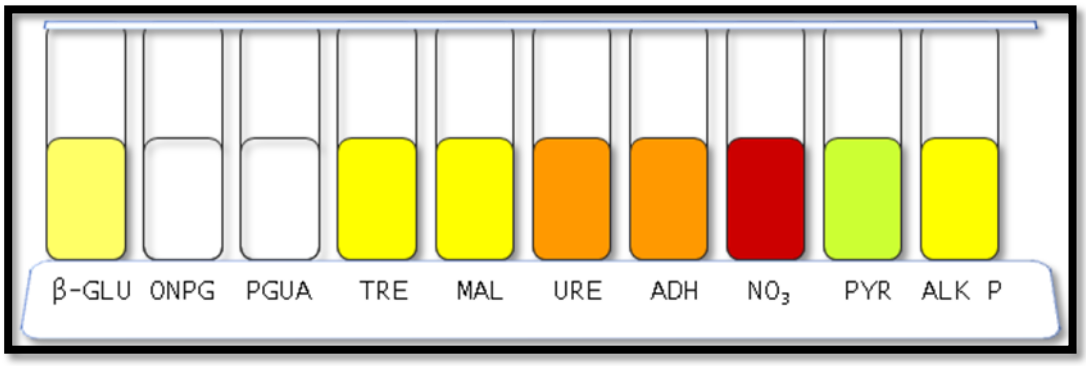

Figure 2.1. Staph-Zym test panel for the different colour reactions for the detection of enzymatic activity. The following enzyme activity was tested: beta-glucosidase (β-GLU), beta-galactosidase (ONPG), beta-glucuronidase (PGUA), trehalose (TRE), maltose (MAL), urease (URE), arginine dihydrolase (ADH), nitrate reduction (NO_3), pyrrolidonyl aminopeptidase (PYR) and alkaline phosphatase (ALK P). The different colour reactions signify whether the test is positive or negative

Table 2.3. Staph-zym record slip for recording of results. Ten enzymatic activity tests are divided into 3 groups of 3 tests and one group with only 1 test. Also included on the result slip was the Natural susceptibility to novobiocin, polymixins and deferoxamine.

Test	**β-Glu**	**ONPG**	**PGUA**	**TRE**	**MAL**	**URE**	**ADH**	**NO_3**	**PYR**	**ALK P**
Values of positives	1	2	4	1	2	4	1	2	4	1
Results	+	0	0	+	+	0	0	+	0	+
Sum of positives	1			3			2			1

	Novo 5	**Polymyxins**	**Deferoxamine**	
Sensitive	1	2	4	**Code number:** 1321-1 **Identification:** *Staphylococcus aureus*
Resistant	0	0	0	
SUM	1	0	0	

The study was continued only with the *S. aureus* strains and the *E. coli* strain that was a representative of a gram negative organism. The identity of the *S. aureus* isolates was confirmed by amplifying the 16S rRNA gene based on the 8F and 1525 region, as this is one of the highly conserved region across evolutionary lines amongst bacteria (Figure 2.2). The PCR products of about 1500 bp were cleaned and sequenced. The sequences obtained were analysed using the BLAST search tool from NCBI and it gave hits with *Staphylococcus aureus* with 99% identity for the strains tested (Table 2.4). The identification of the bacterial strains using the Staph-zym kit and the comparisons of the 16S rRNA sequences on the NCBI database revealed similar results and they confirmed the identity of the strains to be *S. aureus*. The identity of the *E. coli* strain was confirmed previously in the veterinary biotechnology laboratory based on the comparison of 16S rRNA sequences to NCBI database and screening using selective media (Van der Westhuizen, 2010).

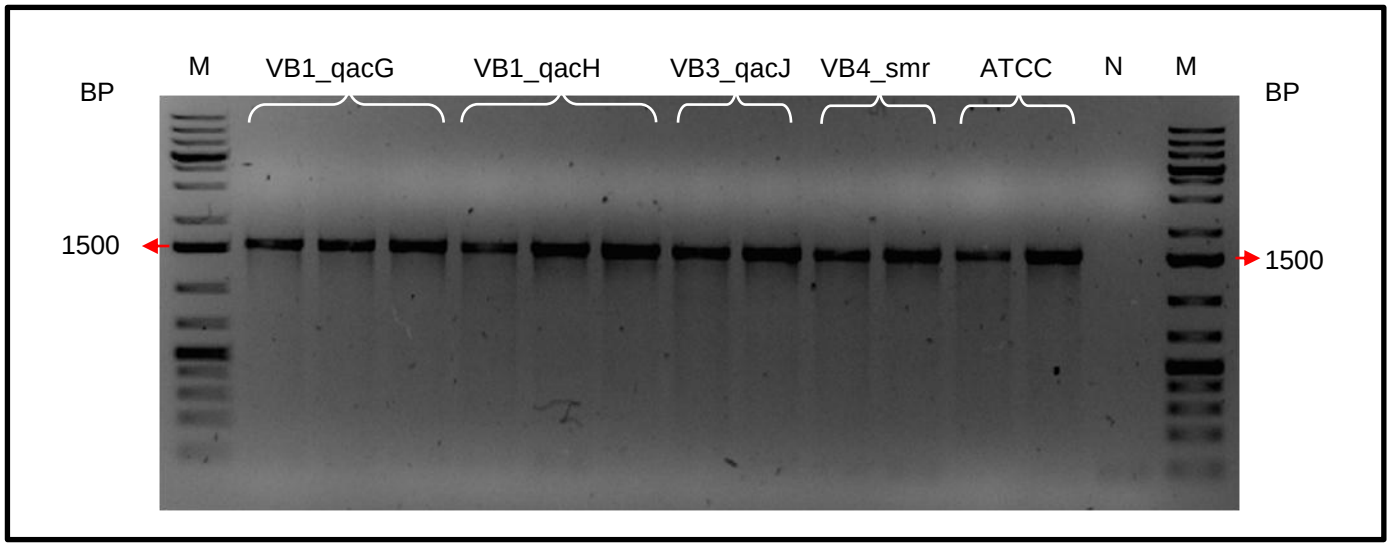

Figure 2.2. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the 16S rRNA gene using primers 8F and 1525R. Strain VB1-qacG, strain VB2-qacH, strain VB3-qacJ, VB4-smr and ATCC 25923. N was the negative and M the marker. The expected band size (~1500 bp) was achieved during amplification.

Table 2.4. Identification of bacterial strains using Staph-zym identification kit and 16S rRNA sequence analysis results.

Isolate	Staph-Zym identification	16S rRNA identification		
		Similarity (%)	Identification based on NCBI 16S rRNA comparisons	E-value
VB1_qacG	*Staphylococcus aureus*	99	*Staphylococcus aureus*	0
VB2_qacH	*Staphylococcus aureus*	99	*Staphylococcus aureus*	0
VB3_qacJ	*Staphylococcus aureus*	99	*Staphylococcus aureus*	0
VB4_smr	*Staphylococcus aureus*	99	*Staphylococcus aureus*	0

2.3.2. Amplification and identification of the QAC resistant genes

The *qac* gene, *smr,* was amplified using colony PCR and a band of the expected size of about 249 bp was obtained (Figure 2.3) and the band was sequenced to confirm its identity. The gene was amplified in the strain VB4_smr as well as in the APEC strain even though this band was not of the expected size. The amplified product obtained for the APEC strain was determined by sequencing. A faint band could be observed for strain VB3_qacJ, but this could not be identified through sequencing. The other *qac* resistant genes, *qacG, qacH* and *qacJ* were amplified in the strains VB1_qacG, VB2_qacH and VB3_qacJ, respectively (Figure 2.4). BLAST results of the sequencing of all of the amplified PCR products for all of the *qac* resistant genes confirmed the identity of the amplified products as the expected genes (Table 2.5). The different *qac* resistant genes could not be amplified in different strains and it would appear that the resistant strains contain only one *qac* resistant gene per strain. In other words, only the qacG gene was found in strain VB1_qacG.

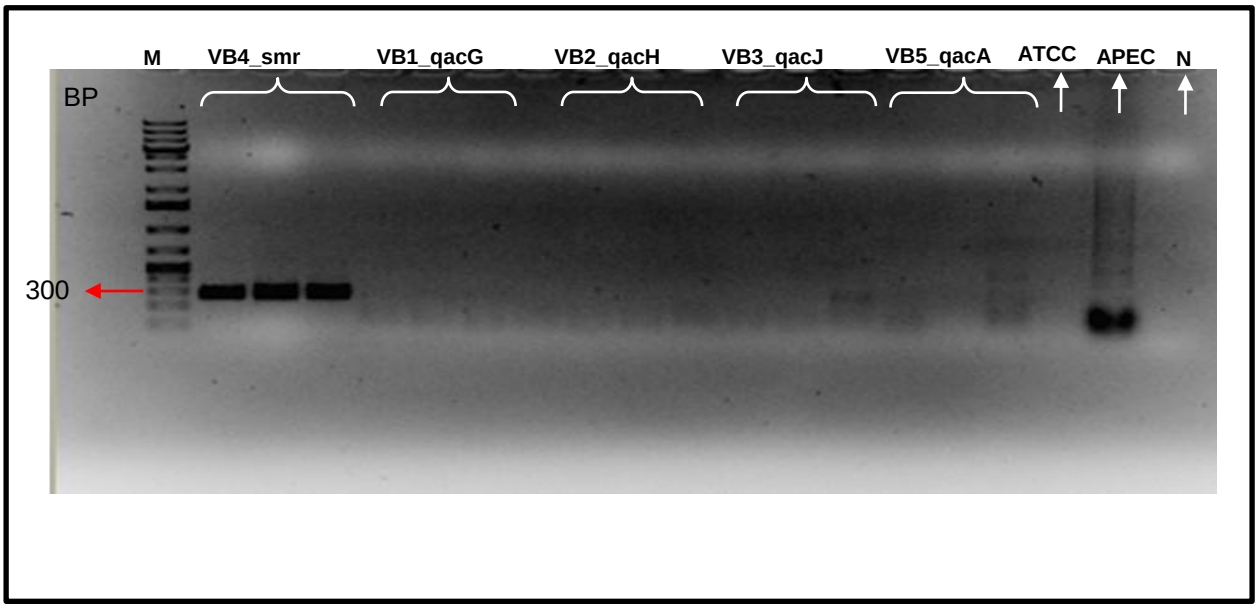

Figure 2.3. A 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the *smr* gene using primers qacC Forw and qacC Rev. The expected band size (~249 bp) was achieved during amplification in strain VB_4 smr. A clear band was also detected in the APEC strain, although this band was not the same size as those seen in VB4_smr.

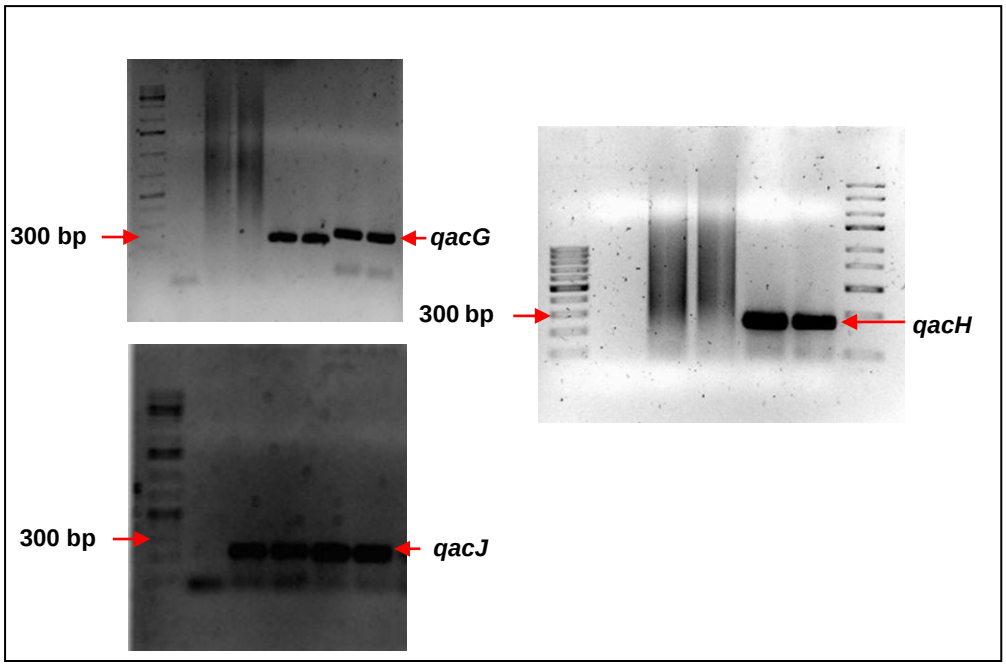

Figure 2.4. 1% w/v agarose gel visualised under UV illumination indicating PCR amplification of the, *qacG*, *qacH* and *qacJ* genes using the primers for the specific genes (Table 2.2). The *qacG* gene was amplified in strain VB1_qacG, the *qacH* gene was amplified in the strain VB2_qacH and the *qacJ* gene was amplified in the strain VB3_qacJ. The approximate band sizes of ~215 bp, 257 bp and 216 bp was achieved during amplification of the *smr*, *qacH* and *qacJ* genes, respectively.

Table 2.5. Blast results for *qac* genes isolated and sequenced (sequences of the strains can be found in Appendix 1).

Isolate	Query coverage (%)	Gene identification with NCBI blast search	Similarity (%)	E-value
VB1_qacG	100	*qacG*	100	0
VB2_qacH	100	(*qacH*) gene, partial cds	100	2E-113
VB3_qacJ	100	(*qacJ*) gene, partial cds	100	9E-38
VB4_smr	100	*qacC* and *qacC'* genes	100	1E-89

2.3.3. Multiplex PCR

A multiplex PCR was developed to randomly screen for bacteria that may have any of the QAC resistance genes present in their genome (Figure 2.5). The expected band sizes for all the genes were similar, between 257 bp and 215 bp, thus the multiplex could not be used as a means of identifying the different genes present in any particular strain, only to identify the presence of at least one or more of SMR genes in an isolate. A single band of just below 300 bp was obtained during multiplex screening of *S. aureus* strains, as well as a faint band was obtained with the strain VB6 where previously, no genes could be amplified. Multiple bands were obtained for the APEC strain during the multiplex amplification (Figure 2.5) and no band was obtained for the susceptible strain, ATCC. This was as expected that only strains resistant to QAC would contain the *qac* genes.

The amplified bands in the multiplex were purified and the different genes were amplified to determine whether the multiplex band observed was a single gene or multiple genes. The resulting products were subsequently sequenced to confirm their identity. The amplified product obtained for the strains VB1_qacG, VB2_qacH, VB3_qacJ and VB4_smr was identified using NCBI blast as *qacG, qacH, qacJ* and *qacC* (renamed *smr*), respectively (Table 2.6). Two amplified products were obtained for the strain VB5_qacA namely *qacH* and *smr*. These products were sequenced and none of the genes' identity could be confirmed using NCBI BLAST (Table 2.6).

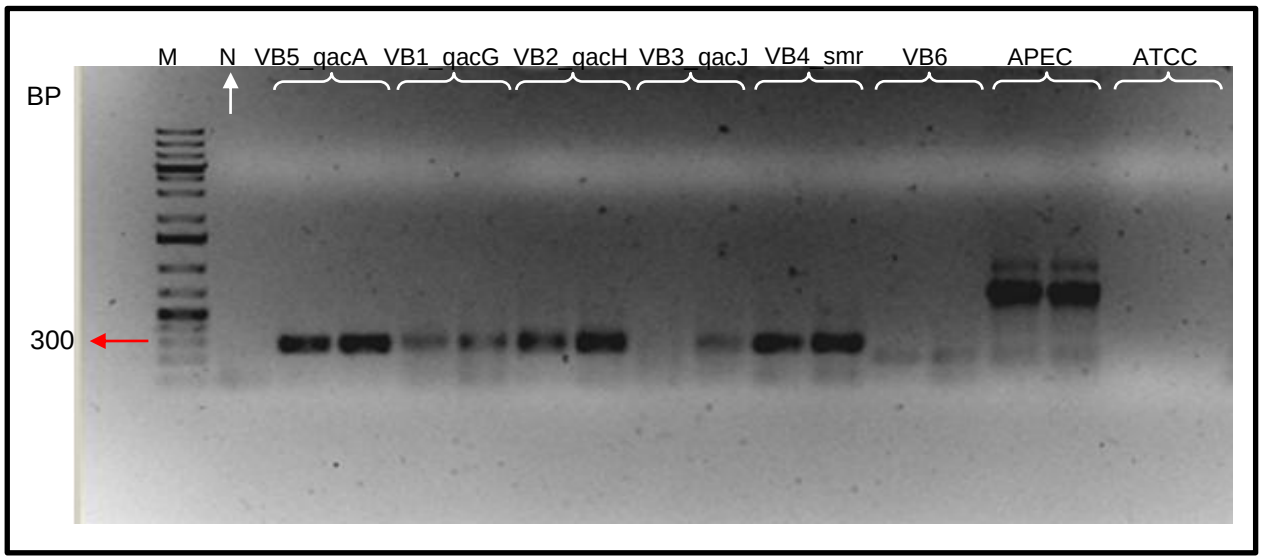

Figure 2.5. 1% w/v agarose gel visualised under UV illumination indicating Multiplex PCR amplification of all *smr* genes: Lane 1 & 2 was strain VB5-qacA, Lane 3 & 4 was strain VB1-qacG, lanes 5 & 6 was strain VB2-qacH, lanes 7 & 8 was strain VB3-qacJ, lanes 9 & 10 was strain VB4-smr, lanes 11 &12 was VB6, lanes 13 & 14 was APEC strain and lanes 15 & 16 was strain ATCC 25923. N was the negative and M the marker. The expected band size (~247 bp) was achieved during amplification.

Table 2.6. Blast results for *qac* genes sequenced obtained from multiplex PCR (sequences of the strains can be found in Appendix 1).

Isolate	Query coverage (%)	Gene identification with NCBI blast search	Similarity (%)	E-value
VB1_qacG	100	*qacG*	100	0
VB2_qacH	100	(*qacH*) gene, partial cds	100	2E-113
VB3_qacJ	100	(*qacJ*) gene, partial cds	100	9E-38
VB4_smr	100	*qacC* and *qacC'* genes	100	1E-89
VB6	96	*qacC gene*	98	1E-77
APEC	98	*qacC* and *qacC*' genes	97	7E-83
APEC	100	Plasmid pST94, *qacG*	98	3E-103

Similarly, two amplified products was also obtained for the APEC strain, *qacG* and *smr* and their identity was confirmed as mentioned above (Table 2.6). Since *E. coli* is a gram negative organism, it is possible that it possesses different size QAC genes compared to *S. aureus* and this could be the reason why multiple bands were observed.

2.3.4. Minimal inhibitory concentration

The Minimal inhibitory concentration (MIC) was determined in order to relate resistance against selected QACs, to the presence of the different *qac* resistance genes. The MIC for the different QACs, benzalkonium chloride (BC), alkylbenzyldimethyl-ammonium chloride (AAC), didecyldimethyl-ammonium chloride (DDAC) and Virukill was determined using the same method used to determine the MIC for antibiotics where the cells are in contact with the QAC for 16 h. This was a very long contact time between the micro-organisms and the disinfectant. The MICs are presented in Table 2.7. For this study, a strain was deemed resistant if it had a MIC of 2 µg ml^{-1} or higher against any of the QACs tested. This value was chosen from literature as the MIC for resistance to benzalkonium chloride (Bjorland *et al.,* 2003).

BC was one of the first QACs developed and all of the strains, including strain ATCC 25923 were found to be resistant to benzalkonium chloride when exposed to the QAC for an extended contact period with MICs of 5 µg ml^{-1} and higher. Strain ATCC was found to be susceptible to the other QACs used in this experiment with MICs below 2 µg ml^{-1}. Strains VB2_qacH and VB5_qacA had a similar MIC for BC as the control strain but had similar MIC for the rest of the QACs as the rest of the resistant strains. The APEC strain had the highest MIC of 10.74 µg ml^{-1} recorded for BC and strain VB1_qacG had the highest MIC recorded for BC 8.79 µg ml^{-1} of the *S. aureus* strains. VB3_qacJ and APEC had the highest MIC recorded for AAC than all the strains. VB1_qacG, VB2_qacH and ATCC were the only strains susceptible to DDAC with MICs below 2 µg ml^{-1} (Table 2.7). The highest MIC recorded for DDAC was 2.93 µg ml^{-1} for the VB5_qacA. All strains were susceptible to Virukill with MICs of 0.703 µg ml^{-1} the lowest MIC recorded was 0.35 µg ml^{-1} for ATCC.

Table 2.7. Minimal inhibitory concentration for strains against the QACs Benzalkonium chloride (BC), alkyldimethylammonium chloride (AAC), didecyldimethylammonium chloride (DDAC) and Virukill with a 16 h contact time.

Strains	BC (µg ml^{-1})	AAC (µg ml^{-1})	DDAC (µg ml^{-1})	Virukill (µg ml^{-1})
ATCC 25923	5.86 (± 0)	1.7 (± 0.55)	0.59 (± 0)	0.35 (± 0)
VB1_qacG	8.79 (± 2.93)	5.86 (± 0)	1.95 (± 0.63)	0.703 (± 0)
VB2_qacH	5.86(± 1.38)	5.86 (± 0)	1.51 (± 0.39)	0.703 (± 0)
VB3_qacJ	7.81 (± 2.76)	8.79 (± 2.93)	2.05 (± 0.88)	0.703 (± 0)
VB4_smr	6.35 (± 2.63)	5.86 (± 0)	2.44 (± 0.63)	0.703 (± 0)
VB5_qacA	5.86 (± 0)	5.86 (± 0)	2.93 (± 0)	0.703 (± 0)
APEC	10.74 (± 2.18)	8.79 (± 2.93)	2.64 (± 0.29)	0.703 (± 0)

During the MIC determination of the strains against the different QACs with a contact time of 20 min, the MIC was significantly higher for all the strains tested (Table 2.8). The highest MIC recorded for BC was 500 µg ml^{-1} for the resistant strains VB1_qacG and VB3_qacJ while the strains VB2-qacH, VB4-smr and APEC had MICs recorded 250 µg ml^{-1}. The strains VB5-qacA and ATCC had low MIC recorded as 31.3 µg ml^{-1} and 15.6 µg ml^{-1} respectively. Similar results were obtained for AAC. The recorded MICs for Virukill was lower than for any of the other QACs, similarly to the MIC recorded for DDAC. The recorded MICs for Virukill and DDAC were lower than for any of the other QACs. The trend in the MICs recorded for a contact time of 20 min was similar to what was seen in for the 16 h contact time.

2.4. Discussion

Quaternary ammonium compound (QAC) resistance genes, *qacG, qacH, qacJ* and *smr* confer resistance to a wide range of quaternary ammonium compound related disinfectants (Paulsen *et al.,* 1995). The SMR, QacG, QacH and QacJ proteins are all structurally similar and all confer resistance via a proton driven efflux system (Heir *et al.,* 1998; 1999; Bjorland *et al.,* 2003). The small multidrug resistance genes also

Table 2.8. Minimal inhibitory concentration for strains against the QACs BC, AAC, DDAC and Virukill determined with 20 min contact time

Strains	BC (µg ml^{-1})	AAC (µg ml^{-1})	DDAC (µg ml^{-1})	Virukill (µg ml^{-1})
ATCC 25923	15.6 (± 0)	7.8 (± 0)	4.9 (± 0)	2.4 (± 0)
VB1_qacG	500 (± 0)	125 (± 0)	19.5 (± 0)	19.5 (± 0)
VB2_qacH	250 (± 0)	62.5 (± 0)	39 (± 0)	19.5 (± 0)
VB3_qacJ	500 (± 0)	31.3 (± 0)	19.5 (± 0)	19.5 (± 0)
VB4_smr	250 (± 0)	125 (± 0)	19.5 (± 0)	19.5 (± 0)
VB5_qacA	31.3 (± 0)	31.3 (± 0)	4.9 (± 0)	2.4 (± 0)
APEC	250 (± 0)	62.5 (± 0)	19.5 (± 0)	19.5 (± 0)

named the *qac* resistance genes are one of the smallest transporters known to actively transport toxins and disinfectants out of the cell, decreasing the concentration of these compounds (Schuldiner *et al.,* 1997; Borges-Walmsley & Walmsley, 2001).

During the study four gram positive resistance strains were obtained and the 16S rRNA gene was amplified and sequenced (Figure 2.3, Table 2.4). The strains were classified as *Staphylococcus aureus* and correlated to the results obtained with the Staph-zym kit and various biochemical tests. *S. aureus* strain ATCC 25923 was obtained to be a representative of a strain susceptible to QAC, thus the control strain. The minimal inhibitory concentration (MIC) for the QACs, benzalkonium chloride (BC), alkyldimethylammonium chloride (AAC), didecyldimethylammonium chloride (DDAC) and Virukill against the selected strains were tested. Virukill was selected as a representative of a commercially available QAC and DDAC because it is the active ingredient in Virukill, BC was selected as a representative of a first generation QAC and AAC as it is an active ingredient in many QACs.

The QAC genes, *smr, qacG, qacJ* and *qacH* were identified in the respective resistant strains (Figure 2.4 and Figure 2.5). The genes were amplified and

sequenced for identification (Table 2.4). Only one gene was amplified and identified in the strains VB1_qacG, VB2_qacH, VB3_qacJ VB4_smr and VB5_qacA but 2 genes were amplified and identified in the strains VB6 and APEC. None of the genes could be amplified from the control strain, ATCC 25923. Multiplex PCR is a useful tool to screen for *qac* genes in field *S. aureus* strains which have at least one of the different *qac* genes. The multiplex seem to be species specific as a different banding pattern was observed during the screening of the APEC strain (Figure 2.7). A possible reason for the different pattern is that gram negative organisms have a complex cell membrane and more proteins are involved in the efflux as these pumps need to pump the QACs through two membranes (Borges-Walmsley & Walmsley, 2001; Putman *et al.,* 2000). *S. aureus* strains were the only gram positive organisms isolated that were resistant to QACs, thus whether the multiplex PCR will work with any other gram positive organism still needs to be tested.

The minimal inhibitory concentration (MIC) against the strains containing the QAC resistant bacteria confirmed the findings by Paulsen and co-workers (1995) that the presence of these genes confer resistance to QACs in these organisms. The control strain ATCC 25923 was resistant to BC even though none of the *qac* genes could be amplified. The MIC for Virukill was below 1 µg ml^{-1} for all the strains thus indicating that all of the strains tested, irrespective of the confirmed presence of a QAC resistance gene were susceptible to Virukill. The MIC for all the strains was determined in a microtiter assay similar to the MIC determination for antibiotics (Figure 2.6). Strains were regarded as resistant to QACs when they had a MIC of 2 µg ml^{-1} or more for any QAC. The strains containing *qac* genes had high MIC for the different QACs tested and displayed a similar profile for most of the QACs.

The ATCC strain was susceptible to all of the different generations of QACs except for BC where it had a MIC of 5.8 µg ml^{-1}. BC was one of the first QACs developed and it has been shown that most bacteria are resistant to this QAC and this was evident in the MIC for the control strain ATCC 25923 of 5.8 µg ml^{-1}. MICs determined during 20 min contact time were higher than MICs determined during contact time of 16 h. The MICs determined for the strain ATCC 25923 against all the QACs tested were below 10 µg ml^{-1}. The MICs determined for the resistant strains against Virukill was 78 µg ml^{-1}, but the highest MIC recorded was 156 µg ml^{-1} for the APEC strain. This was opposite to the findings during a contact time of 16 h where all organisms were susceptible to Virukill (Table 2.7, 2.8). The organisms had the lowest recorded

MIC against DDAC. APEC had the highest MIC recorded against all the QAC during a 20 min contact time.

The MIC recoded for both the 20 min and 16 h exposure times were significantly lower than the concentration generally used in disinfection, but the possibility still remains that the organisms might be exposed to concentrations low enough for resistant clones to survive. The MICs of these bacteria could potentially be much higher in a setting outside the lab as they could grow in conditions such as biofilms that would make them much more resistant to biocides (Campanac *et al.,* 2002). During the growth in biofilms it has been shown that the exopolysaccharide conferred high level resistance to QACs (Campana *et al.,* 2002; McBain *et al.,* 2004). This and the presence of organic material could dilute any QAC used in the disinfection of hard surfaces causing bacteria to adapt to the lower concentrations and become resistant.

The presence of the *qac* resistance genes in strains isolated from poultry is very important, because this is an area where disinfectants and antibiotics are frequently used and organisms may come into contact with both, thus a link between antibiotic and disinfectant resistance is possible.

The ATCC strain's susceptibility to QAC as well as the absence of the *qac* genes resulted in the use of this strain as the control strain. An APEC strain (Van der Westhuizen, 2010) resistant to QACs was isolated from poultry pens and was a representation of a resistant gram negative organism. Gram negative organisms are frequently more resistant to toxic substances than gram positive organisms because of their more complex cell wall (Sidhu *et al.,* 2002). During the multiplex PCR two *qac* genes were identified in the APEC strain and because this strain generally had higher MIC levels to the QACs it could also be a contributing factor to the strains resistance. There is no evidence that the different *qac* genes confer different levels of resistance to the different QACs even though some of the strains did display slight increases in their MICs during long term exposure to the QACs (Table 2.7)

The QAC resistance genes have been identified in different bacterial species, but they may have different sizes and could have different sequences. There are however, conserved amino acids in all the QAC resistance genes that may aid the identification of these genes (Bjorland *et al*., 2003; Paulsen *et al.,* 1995). These genes are also found on mobile elements such as plasmids and transposons, so the possibility that the *S. aureus* genes could be transferred and subsequently detected

in different species is also possible (Bjorland *et al.,* 2005; Heir *et al.,* 1999; Gillings *et al.,* 2009a). Bacteria are able to transfer resistance genes to different strains and thus increase the spread of these genes. The fact that these genes are found on mobile DNA elements also makes them very transient and thus the organism could lose them at any point in time resulting in the organisms becoming more susceptible to the toxic compound. Disinfection is an important factor to general hygiene and it could possibly minimize the use of antibiotics but knowledge on the correct use of these substances is vital to the continued health of poultry.

Chapter 3

The effects of Quaternary Ammonium Compounds on the morphology of *Staphylococcus aureus*

3.1. Introduction

In an attempt to understand how bacteria become resistant to quaternary ammonium compound (QAC) based products, it is important to firstly try to get a clear understanding of how these compounds affect and kill bacterial cells. QACs are cationic detergents and their main target seems to be the cytoplasmic membrane (Hamilton, 1968; Hegstad *et al*., 2010; Ioannou *et al*., 2007; Sandt *et al*., 2007). Membrane active compounds can induce damage on cell membranes and membrane permeability (Al-Adham *et al*., 1998). Attempts have been made to record the morphological changes in bacteria after treatment with QACs; Kourai and co-workers (1994) observed that, what they termed “bleb” formations, on *Escherichia coli* cells, was similar to structures observed by Yohimatsu and co-workers (2007) on *Staphylococcus aureus* cells treated with high concentrations of QAC. In addition, Wattanaphansak and co-workers (2010) observed particle-like debris from bacterial membranes after the treatment with QACs which suggested that QAC based disinfectants might have the potential to lyse bacterial cells (Kourai *et al*., 1994; Yohimatsu *et al*., 2007; Wattanaphansak *et al*., 2010).

It was observed that some unicellular organisms can also die in a way that resembles apoptosis, in this case cell necrosis, where biochemical events lead to unusual cell changes (Benndorf *et al*., 2004), when affected by compounds such as QACs. These changes include blebbing; swelling, bursting and spilling cellular content, but the molecular mechanisms of bacterial apoptosis are not clearly understood (Raff, 1998). One method of apoptosis that has been documented is phage exclusion (Georgiou *et al*., 1998; Gottesman, 1998) where bacterial apoptosis shares similarities with the caspase-dependent apoptosis in animal cells, but it was found that the proteases involved in the processes are unrelated (Raff, 1998). Bacterial apoptosis might be an important factor that could cause the death of cells during antibacterial activity and it could also play an important role in the process that allows bacteria to become resistant to these substances.

Recently, scanning auger microscopy (SAM) was used for the first time in biological samples during the study of the sexual structures of yeasts (Swart *et al*., 2010). Usually, SAM is used for the near-surface analysis of conductors and semi-conductors while the sample is visualised by scanning electron microscopy (SEM) (Hochella *et al*., 1986; Calvo-Bario *et al*., 2001). SAM has also been used to perform in depth studies where the Argon (Ar^+) ion gun has been used for targeted etching on materials such as semi-conductors (Calvo-Bario *et al*., 2001) and more recently biological material during the study of the sexual structures of yeasts (Swart *et al*., 2010). The Ar^+ gun was used to etch through the sample in nanometer thick segments while the SAM and SEM modes were applied to analyse and visualise the elemental composition and 3D ultrastructure of the surface of the cells. Nano scanning auger microscopy (NanoSAM), as this technique is now called, brings a new and powerful means to view and analyse the changes in microorganisms as the SEM mode allows for bigger magnification and resolution of the samples. In this study, NanoSAM was applied for the first time to investigate the effects QACs have on the cells of *S. aureus*.

3.2. Materials and Methods

3.2.1. QAC treatment of cells for scanning electron microscopy

Staphylococcus aureus strain ATCC 25923 (Table 2.1) was cultivated in TSB at 37°C for 16 h and the cells were harvested by centrifugation at 7000 x *g* for 5 min at 4°C, washed twice in 2 mM 3-morpholinopropanesulfonic acid (MOPS, pH 7.2) buffer (Sigma-Aldrich, South Africa) and finally resuspended in MOPS buffer containing 0.4 g l^{-1} and 20 g l^{-1} of the QAC, DDAC (Uniquat 2280, Lonza USA) for 10 min and then prepared for SEM. DDAC was chosen as it was the only non-commercial QAC with the lowest MIC for all strains (Table 2.6 and Table 2.7) under a contact time of 16 h and it is also the active ingredient in Virukill, which showed the lowest MIC (Chapter 2). *S. aureus* strain ATCC 25923 cells used as a control was resuspended in MOPS buffer without DDAC and prepared for SEM in the same manner as described below. Gram stain was performed on the cells and viewed under the light microscope.

3.2.2. Scanning electron microscopy

Cells were fixed for SEM following the same procedure used by Swart and co-workers, (2010). Briefly the procedure entailed the following: Cells were fixed by adding 6% (v/v; 0.1 ml L^{-1}) gluteraldehyde in sodium phosphate buffer and incubated

for 2 h (Merck, South Africa). After incubation, cells were washed with the same buffer to remove excess aldehyde fixative. Post-fixation was performed with 0.5% (m/v) buffered osmium tetroxide and incubated for 1 hour (Merck, South Africa). The cells were washed to remove excess osmium tetroxide. Cells were harvested on 0.2 µm filter and dehydrated by an ethanol sequence 50%, 70%, 95% for 10 min per step and followed by 100% (x 2) for 30 min. Drying was performed by using a critical point dryer. Thereafter specimens were mounted on stubs, coated with gold and viewed with SEM (Shimadzu SSX-550 Superscan, Tokyo, Japan).

3.2.3. Nano Scanning Auger Microscopy (NanoSAM)

The above samples were examined using a PHI 700 Nanoprobe (Japan) equipped with SAM and SEM facilities. The field emission electron gun used for the SEM and SAM analysis was set at: 2.34 A filament current; 4 kV extractor voltage and 238.1 µA extractor current. With these settings a 25 kV, 10 nA electron beam was obtained for the Auger analysis and SEM imaging. The electron beam diameter was 27 nm. The upper pressure of the electron gun unit was 8.8E-10 Torr. The pressure in the main chamber was 2.29E-10 Torr. Aperture A was used for all the measurements. The Field of View (FOV) for SEM was 2.0 µm and the number of frames used was 4. The Auger point analysis were obtained by using 10 cycles per survey, 1 eV/step and 20 ms per step. The nanoprobe was also equipped with an argon ion sputter gun set at: 2kV beam voltage, 2 µA ion beam current and a 1 X 1 mm raster area, giving a sputter rate of 27 nm min^{-1} for the first 3 min, and then reduced to 8.5 nm min^{-1}. The ion emission current was set at 15 mA. An alternating sputter mode with sputter intervals of 1 min and sputter time of 2 min was used without any rotation.

3.3. Results

Didecyldimethylammonium chloride (DDAC) induced morphological changes in *Staphylococcus aureus* cells. The MIC of DDAC against *S. aureus* strain ATCC 25923 was determined previously in Chapter 2 to be 0.59 µg ml^{-1}; consequently the cells were treated with a sub MIC DDAC concentration of 0.4 µg ml^{-1} to observe any possible changes induced by DDAC. The DDAC concentration appeared to be too low and the DDAC concentration was subsequently increased to 20 g l^{-1} which is significantly higher than the MIC value calculated in Chapter 2.

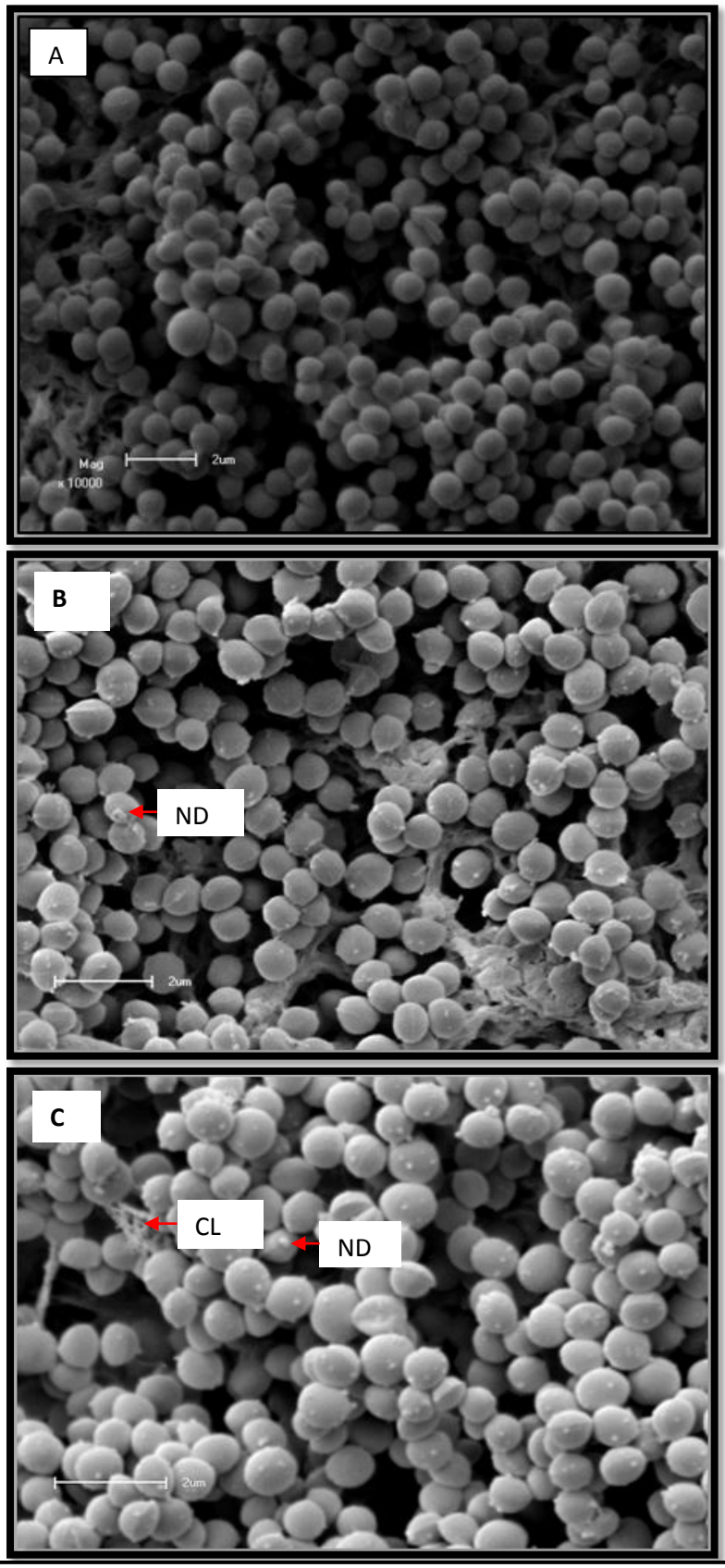

Figure 3.1. Scanning electron micrographs of *Staphylococcus aureus* strain ATCC 25923 (**A**) control cells, (**B**) cells treated with DDAC at a concentration of 0.4 µg ml^{-1}, sub-MIC for this strain, for 10 min, and (**C**) cells treated with DDAC at a concentration of 20 g l^{-1} DDAC for 10 min. Formation of nodules (indicated by arrow) could clearly be visualised in (**C**). ND: nodule on the cell surface, CL: Cell leakage.

The control cells as well as the cells treated with DDAC displayed spherical cells typical of *S. aureus* (Figure 3.1). The cells treated with sub-MIC levels of QAC displayed small nodules on the cell (Figure 3.1 B) that became more pronounced as the QAC concentration was increased to levels considerably higher than the MIC (Figure 3.1 C) and what would appear to be cell material leaking from the cells could clearly be observed in the cells treated with 20 g l^{-1} (Figure 3.1 C). This was not observed in the control and the cells treated with 0.4 µg ml^{-1} DDAC which is an indication that the DDAC affected the cells. Incubating the cells in 20 g l^{-1} DDAC for 10 min was enough to change the gram stain from gram negative to gram positive (Figure 3.2), a clear indicator that the cell membranes were affected by the DDAC. This was an indication that DDAC affected the cell membranes making them porous and allowing the crystal violet to wash out of cells causing them to de-stain and subsequently stain red during the counter staining step in the gram staining procedure and appear as gram negative (Figure 3.2 B).

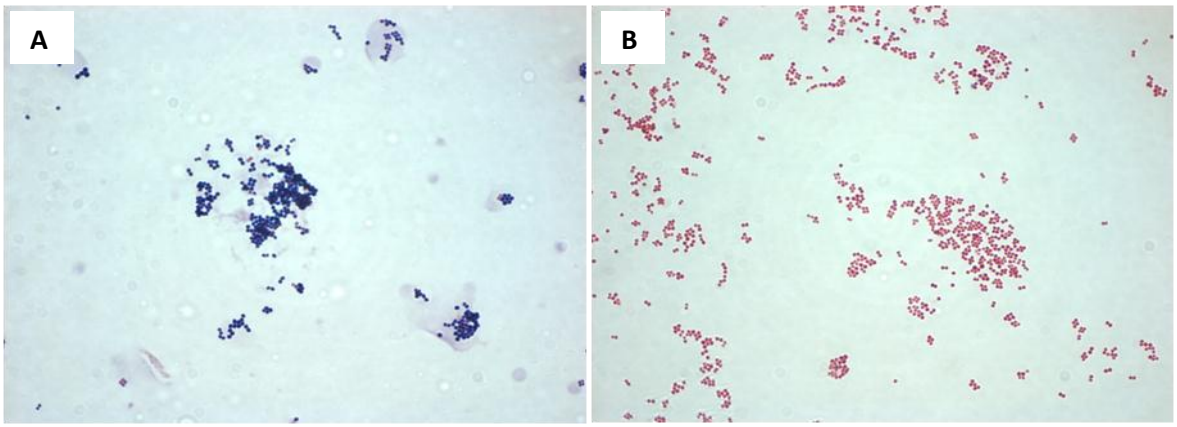

Figure 3.2. Light micrographs of gram stain of *Staphylococcus aureus* strain ATCC 25923 exposed to 20 g l^{-1} DDAC for 10 min. Cells stained the typical blue/purple of gram positive strains before exposure to DDAC (**A**) and changed to gram negative, pink/red after being exposed to DDAC (**B**)

The SEM function of the NanoSAM allowed for great magnification of the bacterial cells (Figure 3.3). The control cells appear smooth with debris on the

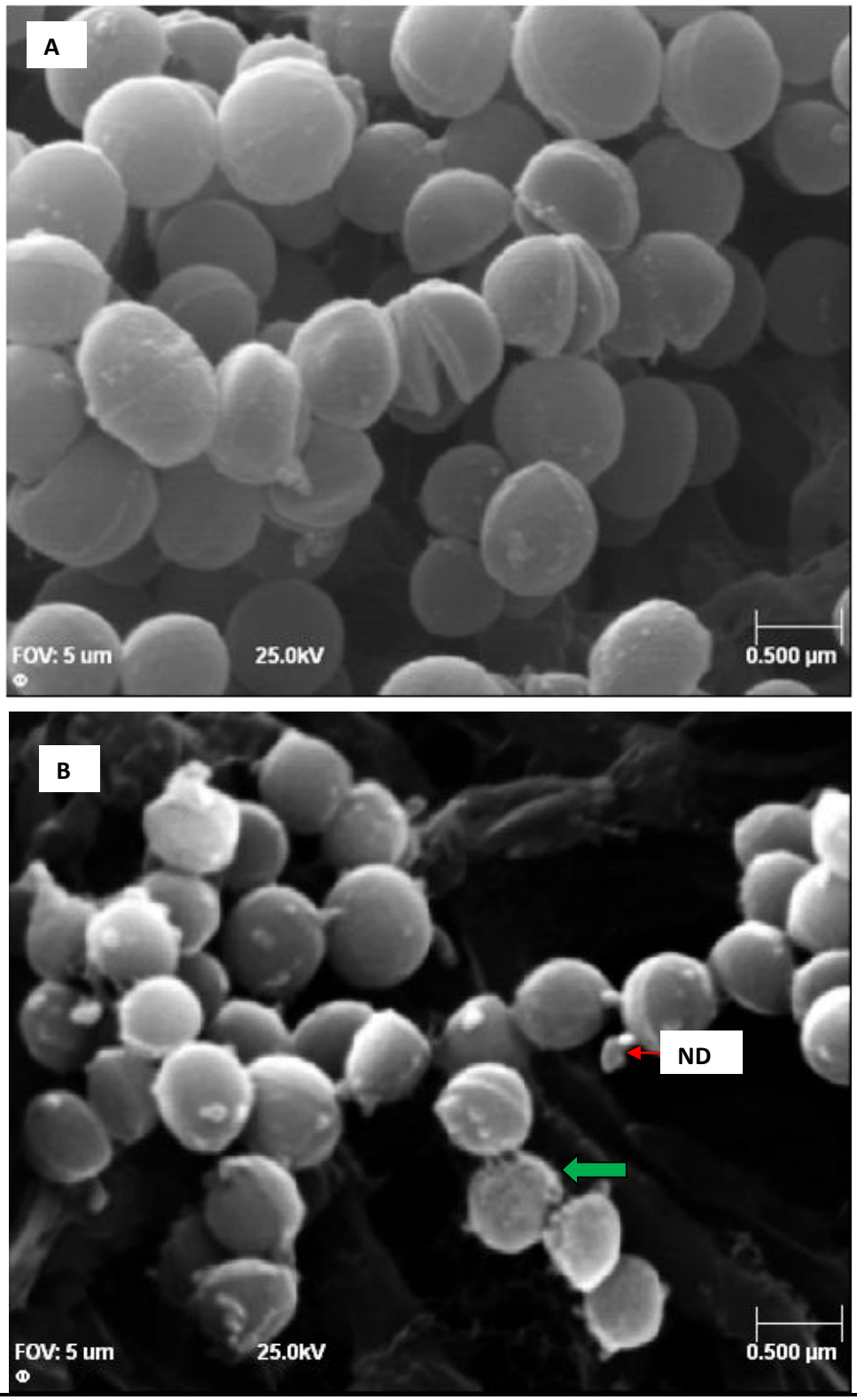

Figure 3.3. Scanning electron micrographs of *S. aureus* strain ATCC 25923cells (**A**) control cells and (**B**) treated with 20 g l^{-1} DDAC. The green arrow indicates a deformed cell.

membranes (Figure 3.3 A). The cells treated with DDAC are deformed and smaller than the control cells (Figure 3.3 B, green arrow). The pronounced nodules on the DDAC treated cells can clearly be seen (Figure 3.3 B). When taking a closer look at the cells, the clear and distinct nodule can be seen (Figure 3.3 B) where on the control cells the nodules are very small and indistinct (Figure 3.3 A). Cells in the process of cell division were also abundant in the control samples (Figure 3.3 A) but not in the DDAC treated cells (Figure 3.3 B).

The ion gun was successfully utilised to produce targeted sputtered etching depth profiles of up to 310 nm into the cells (Figure 3.4). Etching proceeded through the control cells and the cells treated with 0.4 µg ml^{-1} and revealed a smooth layer underneath the gold layer (Figure 3.4 A – F). DDAC concentration of 0.4 µg ml^{-1} did not appear to have any significant effect on the cells. Etching through the cells treated with 20 g l^{-1} revealed a smooth layer and a distinct hole where the nodule was under the gold layer (Figure 3.4 H). Etching was decreased from 27 nm min^{-1} to 8.5 nm min^{-1} in order to improve the visualisation of the cell and internal structures. Etching proceeded straight through the control cells (Figure 3.4 C). The cells treated with 20 g l^{-1} DDAC appeared less stable than the control cells or the cells treated with 0.4 µg ml^{-1} DDAC as the image registration failed due to severe image shift after sputtering.

SAM elemental colour maps indicated a high gold intensity (Figure 3.4 J, gold indicated as green, corresponding to Figure 3.4 G) as the sample was coated in gold during preparation for SEM to make the sample electro-conductive. As etching proceeded, carbon and oxygen were revealed (carbon indicated in blue and oxygen indicated in red; Figure 3.4 K corresponding to Figure 3.4 H). Carbon intensity gradually increased as etching continued, but some gold still remained as the ion gun could not reach these sections of the cells (Figure 3.4 L corresponding to Figure 3.4 I). In Figure 3.5 a cell with a clear nodule with what appears to be cellular material leaking from the cell could be observed. During the etching of another nodule a hole could be observed where the nodule was, but not as distinct as previously (Figure 3.5 C). This indicates that the QAC, DDAC, affected the membranes of the bacterial cells tested in this study.

During the sputtering experiment an auger point analysis spectrum was taken at specific sites on the bacterial cells. Each element has a specific peak profile according to its kinetic energy (Figure 3.6) and this is used to calculate the atomic

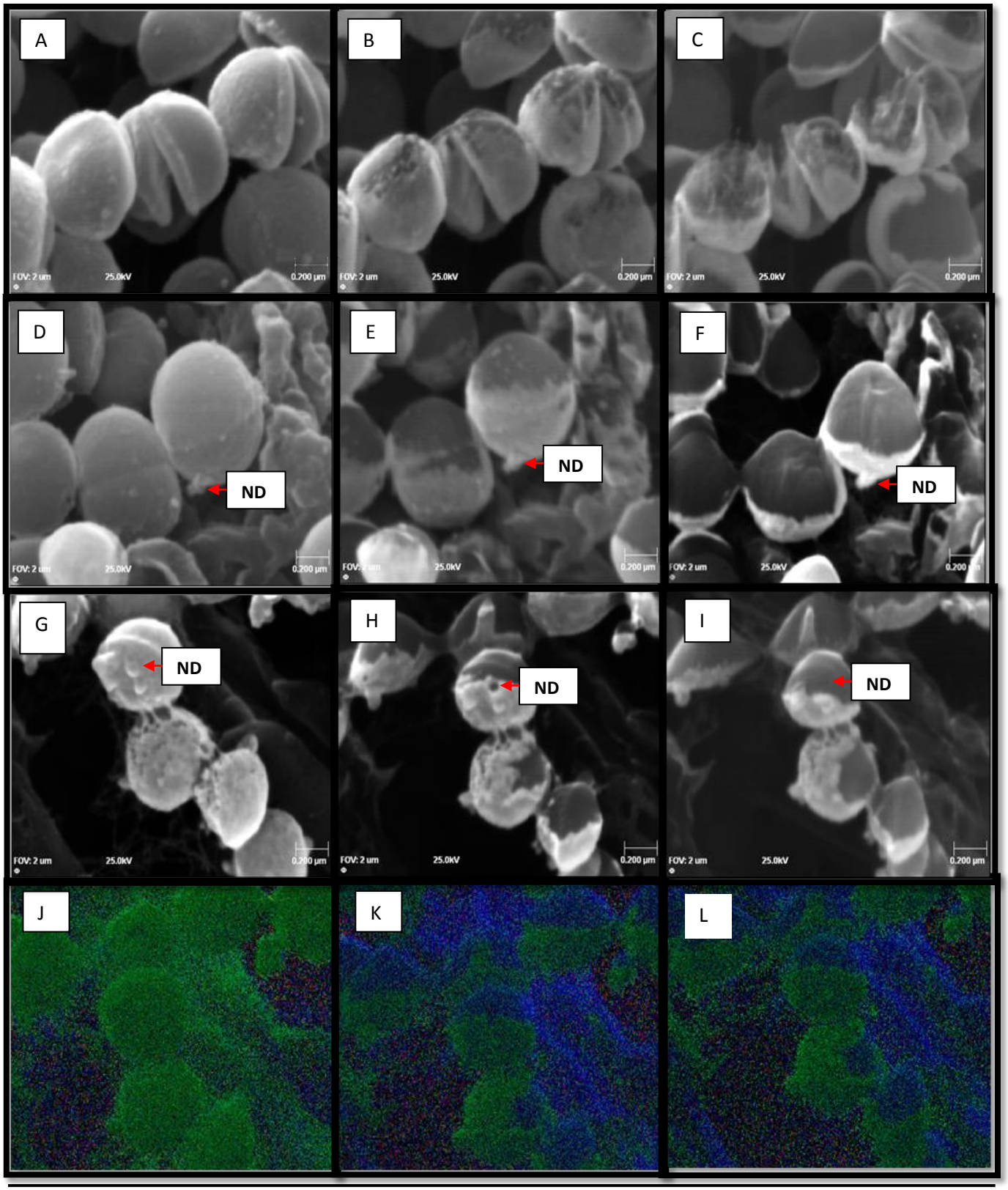

Figure 3.4. Scanning micrographs at different stages of sequential etching into *S. aureus* strain ATCC 25923 control cells without DDAC (a-c), cells treated with 0.4 µg ml^{-1} DDAC (d-f), cells treated with 20 g l^{-1} DDAC (g-i). Colour maps of the various elements present in the sample (j-l). These maps correspond to the etching micrographs (g-i). Green – gold, blue – carbon and red – oxygen. The arrow ND indicates the presence of the nodule on the cell surface

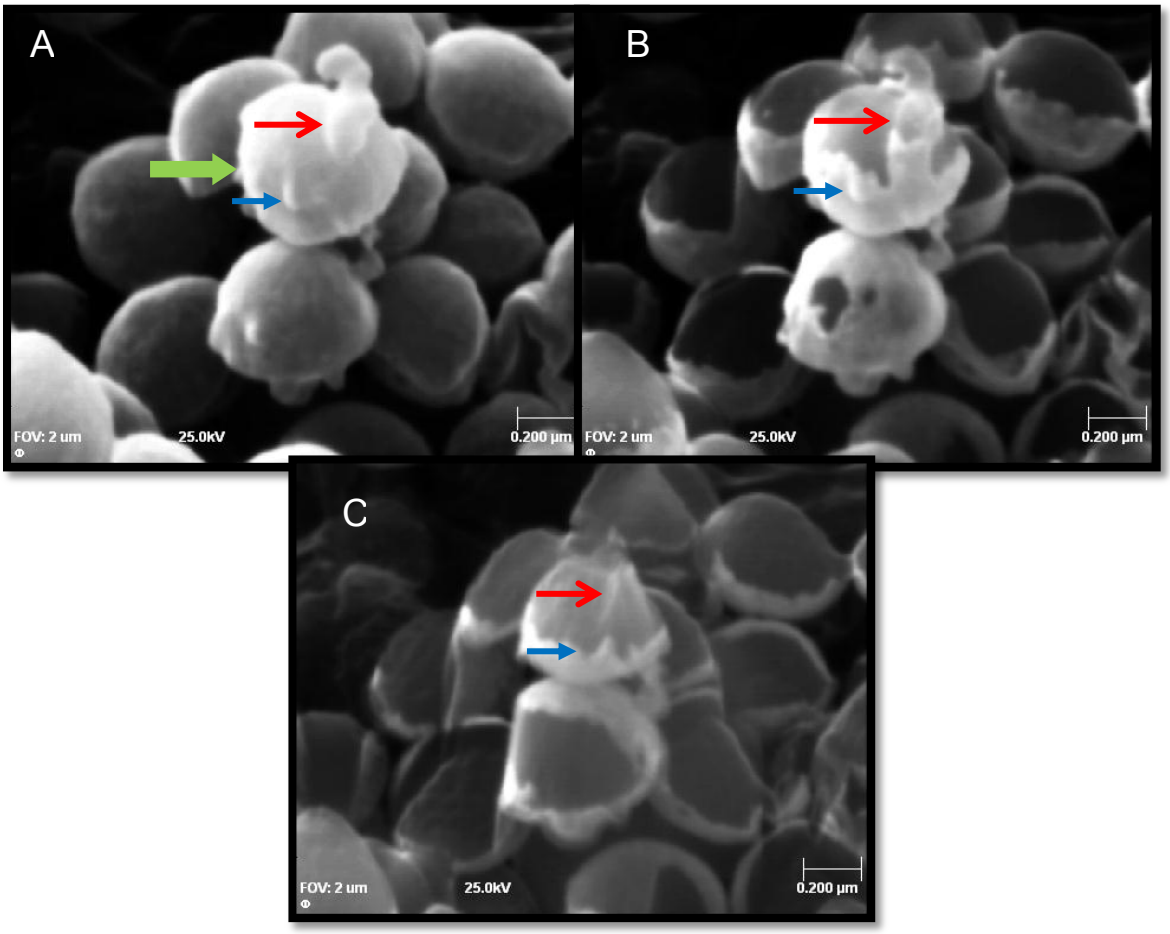

Figure 3.5. Scanning micrographs at different stages of sequential etching into *S. aureus* strain ATCC 25923 control cells treated with 20 g l^{-1} DDAC. Green arrow indicates a deformed cell. Red arrow indicates a nodule which appeared to be cellular material leaking out of the cell and its progressive etching. Blue arrow indicates a nodule and the progressive etching revealing a hole in the cell.

percentages of each element. Elemental composition depth profiles of cells were obtained to make quantitative assessments of the elemental composition as etching proceeded through the cells taken at specific targets on the cells (Figure 3.7 and 3.8). A sharp decrease in the intensity of gold was evident in the control cells followed by a sharp increase in Carbon (Figure 3.7, Target 1). As etching proceeded through the cells some of the other elements associated with the cells, oxygen, nitrogen and osmium started to appear. Osmium was used in the preparation of the cells for SEM and even though the concentration was very low the presence thereof is an indicator that etching proceeded through cell membrane as osmium accumulates in the cell membranes. The other elements remained very low. The profile was similar for all the target sites (Figure 3.7, Target 2 and Target 3).

There was a gradual decrease in the gold concentration of the DDAC treated cells (Figure 3.8 Target 1) with a corresponding gradual increase in carbon at the specific sites. This could possibly be an indication that the cell wall of the DDAC treated cells was not smooth and this might be the reason of the gradual decrease in gold. The cell topography is different and the gold is gradually removed because of the irregularity of the cell wall as a result of DDAC treatment as seen in Figure 3.8. The profile was similar for all target sites indicating that the nodules are part of the cell wall and not foreign material as Target 3 was placed on a nodule. Only trace amounts of chlorine (indicates DDAC as the chemical structure contains chlorine) were detected but it was too little to distinguish it from the background noise to be conclusive (Figure 3.7 Target 1 to 4).

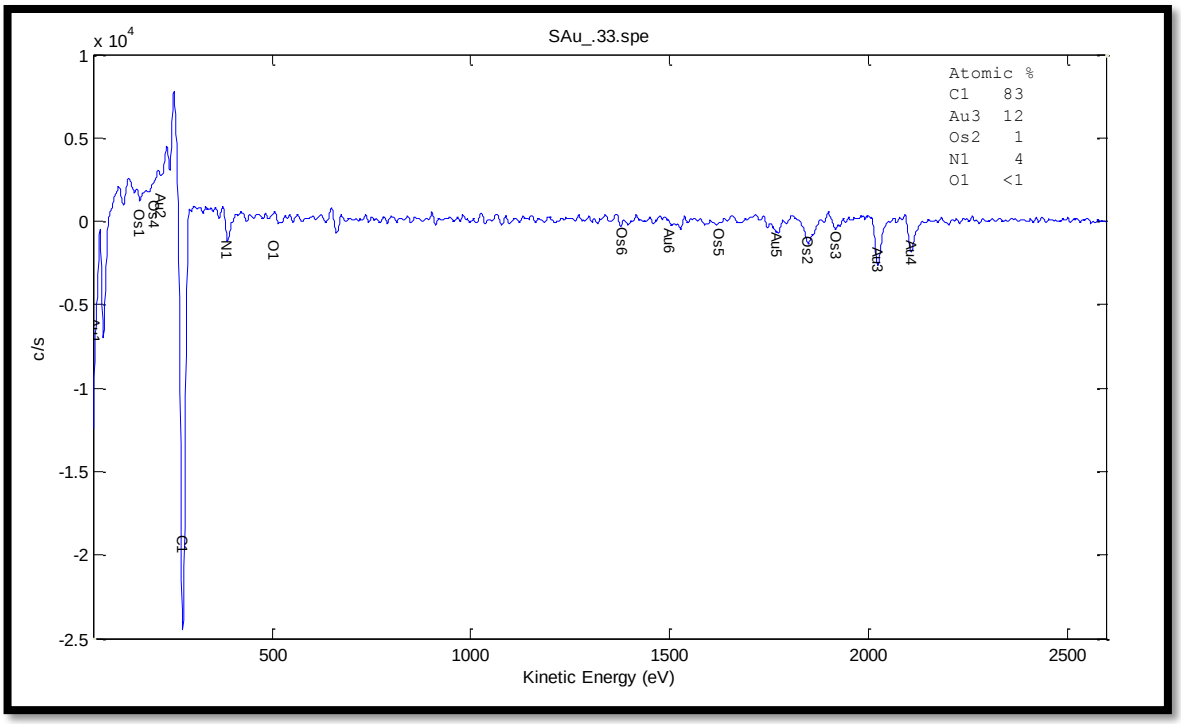

Figure 3.6. Auger point analysis spectrum taken after 33 minutes of sputtering.

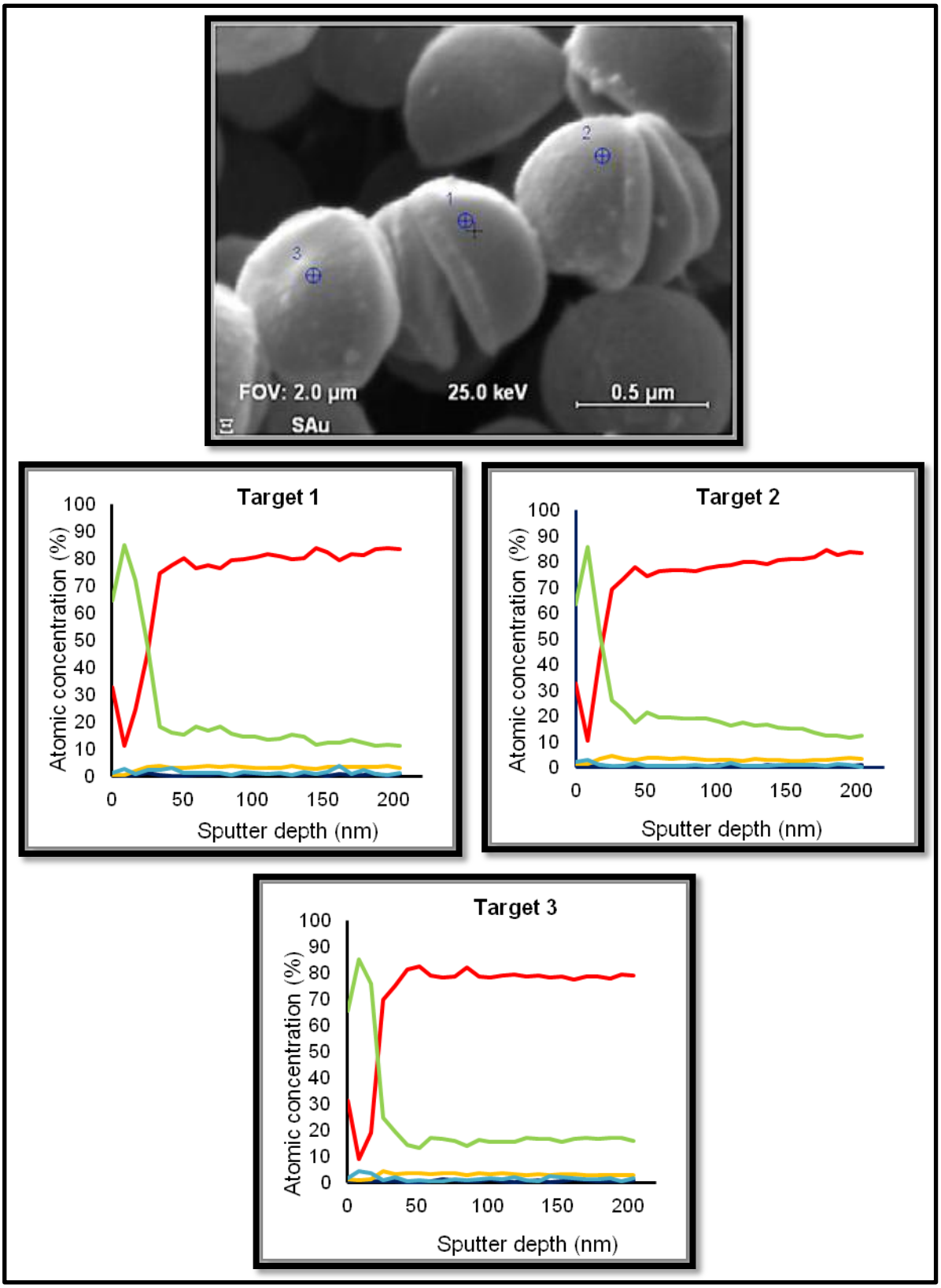

Figure 3.7. Elemental analysis through cells during sequential etching. (a) SEM micrograph of control cells, (c) SEM micrograph of DDAC treated cells, (b) a graph showing atomic concentration over sputter depth of control cells in (a) (target 3), (d) DDAC treated cells (target 2). – carbon, – oxygen, – gold, – nitrogen, – osmium

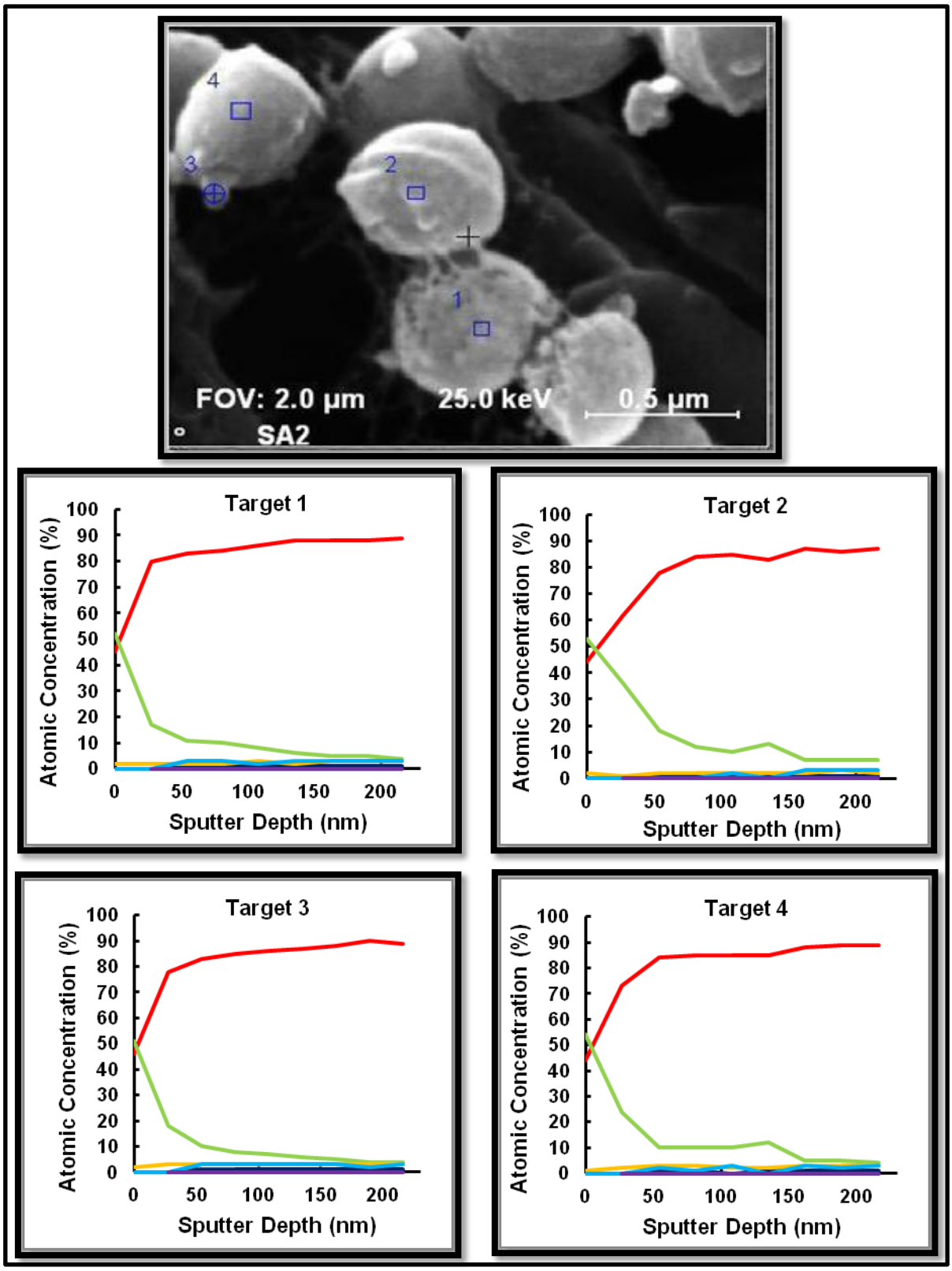

Figure 3.8. Elemental analysis through cells during sequential etching. (b) SEM micrograph of DDAC treated cells, (1b) a graph showing atomic concentration over sputter depth of control cells in (a) (target 3), (d) DDAC treated cells (target 2). –carbon, –oxygen, – gold, – nitrogen, – osmium, – chlorine

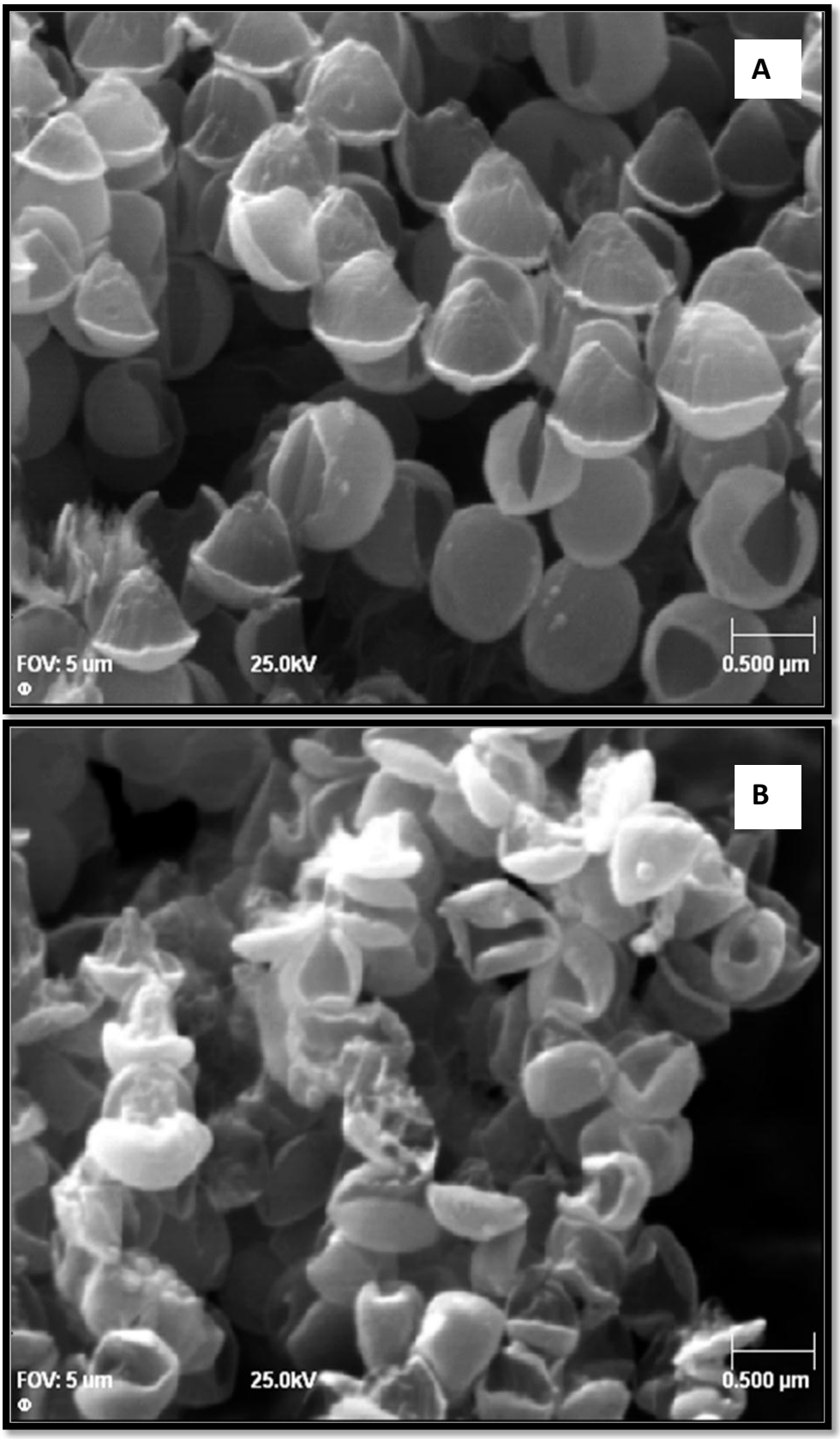

Figure 3.9. SEM micrograph of *S. aureus* strain ATCC 25923 control cells (**A**) and DDAC treated cells (**B**) after 30 min of etching. The DDAC cells completely collapsed under the 25 kV of the argon gun.

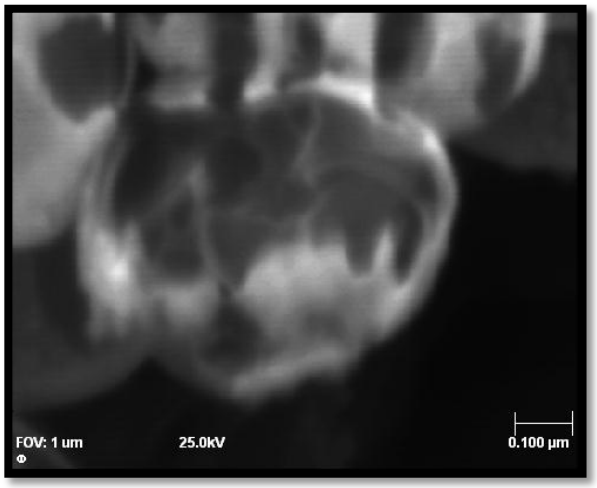

Figure 3.10. SEM Micrograph of strain ATCC 25923 cells treated with 20 g l^{-1} DDAC after 56 minutes of sputtering indicating distinct internal structure seen as light and dark sections. Arrow indicates an internal structure.

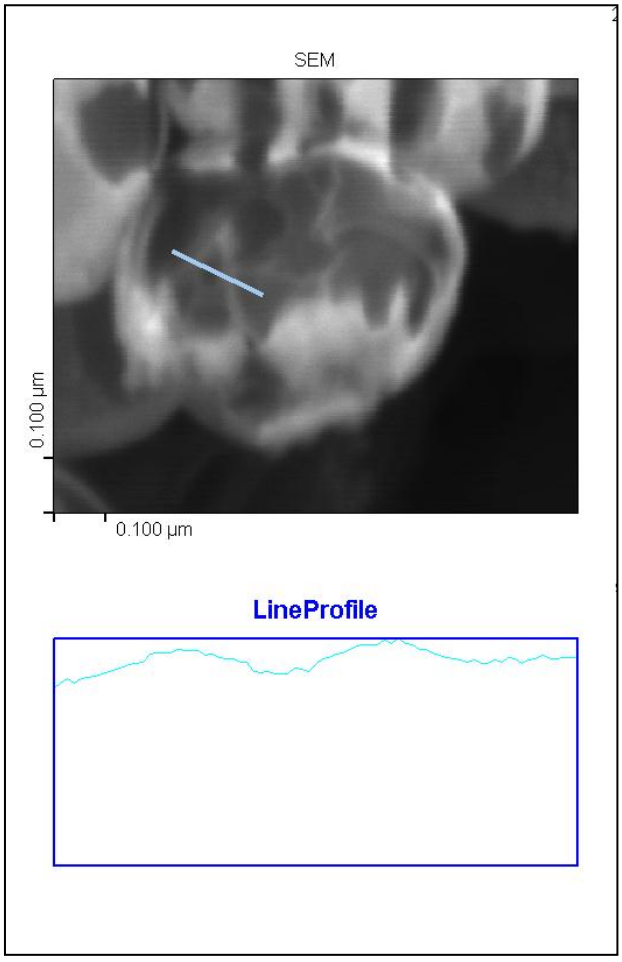

Figure 3.11. Line profile drawn of internal structures visualised with SEM, indicated by the light blue line, to reveal the topography of the exposed section of the cell. The line profile revealed that the dark areas were deeper into the cell than the light parts in effect a hole or channel.

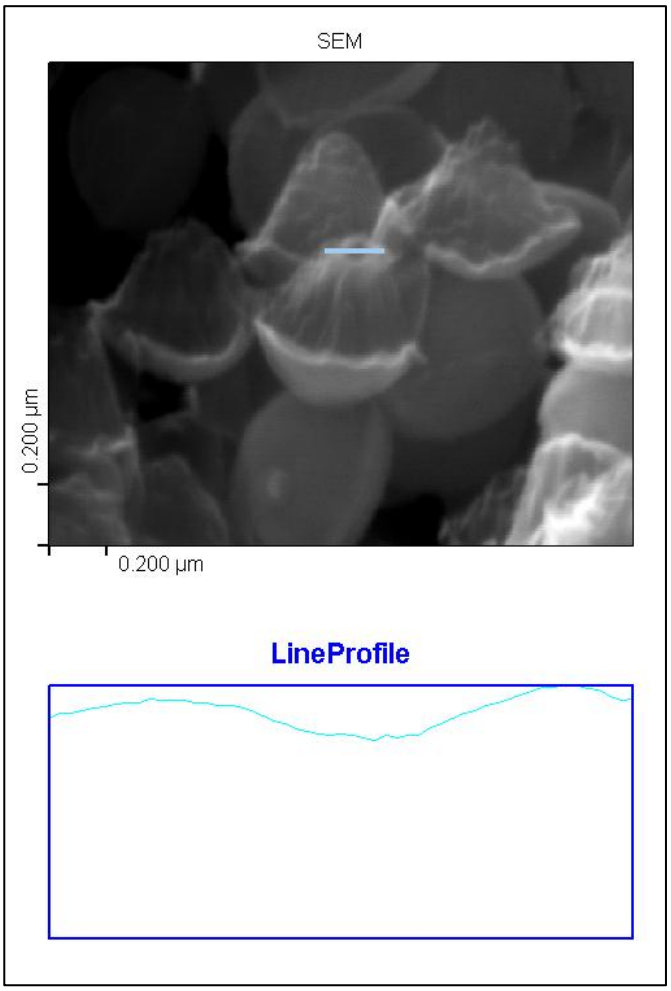

Figure 3.12. Line profile drawn from ATCC 25923 cells treated with 20 g l^{-1} DDAC where a channel of hole in the top of the cells was observed. The line profile indicated that there was a difference in the surface and what appears to be a hole in the cell.

Some of the cells revealed what seemed to be internal structures (Figure 3.10) indicated with the different levels of light and dark areas. Light areas also reveal the presence of gold. This is possibly gold seeping through pores, or it is electron backscatter as etching proceeds. A line profile was drawn on part of the internal structures using software from the NanoSAM to reveal the topography of the cell surface (Figure 3.11). The line profile indicated that this area was not smooth and it is possibly different cell structures that were observed. This function was also used to draw the topography of etched cells where a channel or pore was revealed after etching (Figure 3.12). The line profile revealed a depression on top of the cells. This could possibly be as a result of the DDAC treatment on the cells causing holes and

pores in the cell surface. The cell surface also appeared rough and not smooth as was stated in previous examples.

3.4. Discussion

Scanning Auger microscopy allows for the semi-quantitative elemental analysis on extremely small volumes (Hochella *et al*., 1986). SEM nano-etching with an Argon gun and also elemental analysis using a nanoprobe was previously performed on yeasts (Swart *et al*., 2010). This technology has now been successfully applied on bacterial cells of *Staphylococcus aureus* for the first time. Nano-etching was achieved by sputtering the sample with ionized argon atoms, thereby peeling 8.5 nm segments from the sample after every electron sputtering under vacuum and high pressure. This enabled the detection of the 3-D nanostructure of cells and DDAC-treated cells.

Treatment with DDAC caused the formation of nodules on the cell walls of the cells and possibly caused the leakage of the cellular material (Figure 3.1 C). In literature it has been theorised that detergents, and in particular QAC based detergents cause disorganization of the cell membrane (Salton, 1951). Later, Yoshimatsu and Hiyama (2007) observed bleb formation on *Escherichia coli* cells after treatment with didecyldimethylammonium chloride, and Wattanaphansak and co-workers (2010) observed the particle-like debris from bacterial membranes form on the cell membranes after treatment with disinfectants. During the current study, the same morphological changes were observed (Figure 3.1 and 3.3).

The formation of the blebs or particle-like debris on the cells has not been identified. This study would be the first study where the elemental composition of these ‘blebs’ or nodules was analysed. Sequential etching through a bleb revealed a hole or pore in the membrane that was not observed in the untreated cells where elemental colour maps did not indicate the presence of any element that was foreign to the cell membrane (Figure 3.4). The same trend was seen when etching through more nodules (Figure 3.5). Elemental analysis was performed by focusing the nanoprobe on a specific target site on the sample, and bombarding it with electrons from the nanoprobe resulting in the release of Auger electrons that were detected via a detector and a specific energy profile was obtained (Calvo-Barrio *et al*., 2001). These profiles were specific for every element and asince all elements have a unique set of electron-binding energies and a unique point analysis spectrum (Figure 3.6) the unknown elements in the sample could be identified (Hochella *et al*., 1986). SAM was

achieved by mapping the various elements across the etched surfaces in the sample indicating specific elements in specific colours. A colour map of various elements was obtained for each sample surface analysed (Figure 3.4j - l). DDAC treatment on the cells also made the cells more sensitive and disturbed the balance of pressure within the cell. This made etching through these cells difficult so that after each pelting with electrons, the cells shifted and image registration for SEM was a major problem.

Scanning auger microscopy (SAM) applications generally are involved in the near-surface analysis of conductors and semiconductors (Calvo-Bario *et al.,* 2001; Hochella *et al.,* 1986) and because bacterial cells are biological specimens and are not conductors, it was necessary to make them conductive by sputter coating them as is the case for electron microscopy. As etching proceeded the removal of gold from the surface by Argon etching could be clearly visualized. Elemental composition depth profiles of cells were obtained to make quantitative assessments of the elemental composition as etching proceeded (Figure 3.7). A decrease in gold was evident in both the control cells and the QAC cells (Figure 3.7 and 3.8).

The carbon concentration increased for both these cells, but no significant difference in the elemental composition depth profiles between the different targets in the samples was observed (Figure 3.7 and 3.8) and was clearly different between the control and the DDAC treated cells. It would appear as if the carbon concentration increase gradually in the DDAC treated cells compared to the control cells. This could possibly indicate that the cell surface of the treated cells were uneven compared to the smooth cell surface of the control cells. The depth profile method was capable of determining the elemental composition of the cells and revealed that there was no difference between the elemental composition of the cell membrane or the nodule, indicating that it was part of the cell membrane or cellular material and not the culture medium.

The appearance of osmium indicated that the etching proceeded through the cell membranes, as osmium was used in fixing the cells for SEM and thus accumulates in the cell membranes. QACs are membrane active compounds and thus it would be expected that once etching reached the cell membranes, QAC would be detected. QAC contained chlorine and thus the appearance of chlorine would indicate the presence of QACs. Only trace amounts of chlorine could be detected in the treated cells and no chlorine was detected in the control cells. This was expected as no DDAC was present in the control cells. However, the concentration of chlorine was

too low to definitively conclude that chlorine was present above the background elemental noise. This raises questions as to what happened to the QAC. A possible reason could be that the chlorine is cleaved from the QAC molecule upon entering the cell membrane and was washed away during the steps in preparation for SEM. Another possibility could be that the QAC was modified by the cell and thus it could not be detected. QACs contain a hydrophilic quaternary ammonium group and a hydrophobic alkyl chain (Sandt *et al*., 2007). What the function of the halide (chlorine) is in the disinfection is unknown. This should be further investigated and as to what happened to the chlorine and thus the QAC upon entering the cell.

QACs are membrane active compounds and any membrane active compound could potentially induce damage on the membrane (Hamilton, 1968; Hegstad *et al*., 2010; Sandt *et al*., 2007). QAC causes cell death by the leakage of metabolites and the formation of the nodules could possibly precede the leakage of the cellular material. Yoshimatsu and Hiyama (2007) found that the bleb formation was only seen at higher concentrations of DDAC, but the results in this study (Figure 3.1) the nodule formation was seen at concentrations as low as 0.4 µg ml^{-1}. No nodules were seen on the control cells in the absence of DDAC (Figure 3.3 and 3.4), this indicates that the nodule formation is associated with the presence of DDAC but only at the higher concentrations did it affect the cells negatively. The same was concluded by Yoshimatsu & Hiyama (2007).

Bacterial apoptosis or the leakage of cellular material has been demonstrated in this study where empty cells were observed when cells were treated with DDAC (Figure 3.9). Bacterial apoptosis might be an important factor that could cause the death of cells during antibacterial activity and it could also play an important role in the process that allows bacteria to become resistant to these substances. The cells themselves where damaged to the extent that when the high pressure and cellular bombardment from the electron gun was applied the cells completely collapsed, something that was not observed in the control cells (Figure 3.9).

Another useful function of the NanoSAM system was the ability to draw line profile to reveal the topography of the cells. Line profiles were drawn for cells that appeared to reveal internal structures (Figure 3.11) and cells that appeared to have pore formations (figure 3.12). There were clear light and dark areas in the cell that indicated the different levels of depth within the cell. This gave the appearance of the internal structures seen in Figure 3.11. The line profiles revealed the appearance of a depression in the cell surface Figure 3.12. The cell surface appeared uneven,

something not observed in the control cells, and could account for the difference in the elemental analysis profile for the different cells (Figure 3.7 and 3.8). The gold concentration never drops below a detectable range, but this could be due to backscatter of the electrons as etching proceeds.

It is clear that DDAC affects the morphology of the bacterial cells causing nodule formation and the formation of pores in the cell membrane. Gilbert and Moore (2005) and many other researchers suggested that the QAC penetrates the cell membrane and there cause the leakage and destabilization of the membrane and internal cellular environment. No evidence to support the penetration of cell membranes was found as no detectable chlorine (indicator for DDAC) was found in the membranes or anywhere else in the cell. Further investigation is needed to determine what happens to the QAC after it penetrates the cell.

Chapter 4
The mechanisms of bacterial resistance against Quaternary Ammonium Compounds

4.1. Introduction

Bacteria have the ability to develop increased tolerance to a wide range of toxic compounds including disinfectants through the expression of multidrug resistance genes such as the quaternary ammonium compound resistance genes (*qac*) that code for the SMR efflux pumps (Smith *et al*., 2008;). The presence of *qac* genes possibly results in isolates with a selective advantage when challenged with disinfectants containing QACs (Smith *et al*., 2008). Smith and co-workers (2008) showed that the expression of *qacA/B* genes increased in the presence of disinfectants. The repressor, *qacR* binds upstream form the *qacA/B* promoter overlapping the transcription start site, in the absence of disinfectants and regulates the expression of the *qacA/B* genes (Smith *et al.,* 2008). Similarly, overexpression of the multidrug efflux pumps, *mepA, mdeA, norA* and *norC* has been found in the presence of disinfectants (Huet *et al.,* 2008). Currently the expression of the *qac* resistance genes has not been investigated.

The expression of genes can be determined through the use of relative quantification where the expression of the gene is measured as a ratio of an external standard or a reference sample (Wong & Medrano, 2005). In relative quantification results are expressed as a ratio between the gene of interest and the reference, where the reference is usually a gene that has stable expression (Wong & Medrano, 2005; Theis *et al*., 2007). Usually, housekeeping genes (genes needed for the cell maintenance or a constitutively expressed gene) are used as reference genes (Theis *et al*., 2007). Samples often differ and to compensate for the variation between samples in order to compare the samples, normalization of the gene expression is used to correct for the variation between samples (Pfaffl, 2001; Wong & Medrano, 2005; Theis *et al*., 2007). Normalization is achieved by the ratio between the gene of interest and the reference sample.

Roche LightCycler software incorporates a control sample, called the calibrator, to normalize the samples (Roche Molecular Biochemicals Technical Note No. LC 6/99). The calibrator is a sample of known concentration that contains the gene that will be quantified (the gene of interest). In this case normalization is obtained by dividing the normalized ratio of the gene of interest expressed in the calibrator by the normalized ratio of the gene of interest expressed in the sample being tested. In this chapter, a real time PCR assay was designed to detect the *qac* genes in different bacterial strains. Recently, it was shown that the *qac* genes can be found on class 1 integrons which is widely spread in the natural environment (Gillings *et al*., 2009 a; b). A quantitative real time PCR was also developed to look at the expression of the *smr* gene under conditions of varying QAC concentrations.

4.2. Material and Methods

4.2.1. Cultivation and Quaternary Ammonium Compound treatment of cells for real time PCR

Staphylococcus aureus quaternary ammonium chloride (QAC) resistant strains, VB3_qacJ, VB4_smr, and the QAC susceptible strain ATCC 25923 (Table 2.1) were cultivated in TSB for 18 h. These were used to inoculate tryptic soy broth (TSB) containing different concentrations of didecyldimethylammonium chloride (DDAC); no DDAC, 0.2 µg ml^{-1} DDAC and 1 µg ml^{-1} DDAC for 12 h. RNA was extracted from these cells using the extraction methods described below (4.2.2) and used for qualitative real time PCR.

In addition, *S. aureus* QAC resistant strains VB1_qacG, VB2_qacH, VB3_qacJ, VB4_smr, the QAC sensitive strain ATCC 25923 as well as the APEC strain was cultivated in TSB for 16 h. RNA was extracted from these cells and used for qualitative RNA detection of all the *qac* genes.

4.2.2. Total RNA isolations

During the study, two methods were used for the isolation of total RNA from *Staphylococcus auerus* QAC resistant strains and the susceptible strain cultivated as described above. The total RNeasy mini kit (Qiagen) as well as a modified method for the total RNA extraction method described by Labuschagne and Albertyn (2007) was used to extract RNA from these *S. aureus* strains.

S. aureus cells were harvested in 50 ml centrifuge tubes by centrifugation at 7000 x *g* (4°C) for 1 min followed by total RNA extraction using tri-reagent chloroform method described by Labuschagne & Albertyn (2007). The cells were resuspended in 2 ml Tri-reagent (Sigma-Aldrich, South Africa) containing 1.5 ml glass beads (Sigma-Aldrich, South Africa). The glass beads added to the tri-reagent in breaking and denaturing the cell walls. Cells and glass beads were mixed vigorously using a vortex mixer for 1 min followed by cooling on ice for 1 min. This was repeated for a total of 5 min and incubated at room temperature for 5 min. Following the incubation at room temperature, 400 µl chloroform was added and mixed on a vortex mixer for 15 sec and then incubated at room temperature for 3 min. The mixture was centrifuged at 12 000 x *g* for 15 min at 4°C. After incubation, 3 layers were observed, a pink layer of tri-reagent at the bottom, a chloroform layer containing the proteins and an aqueous layer at the top containing the RNA. The top layer was carefully removed and added into a 1.5 ml eppendorf containing 500 µl isopropanol (Merck, South Africa) and mixed by vortexing and incubated for 10 min at room temperature to allow for the RNA to precipitate. The Isopropanol mixture containing the RNA was centrifuged for 10 min at 12 000 x *g* at 4°C. The RNA pellet was washed with 1 ml 70% ethanol and allowed to dry at room temperature for 5 min. The RNA was resuspended in 50 µl nuclease free water and stored at -80°C.

The second method used was the extraction of RNA using the RNeasy® Mini Kit (Qiagen). Total RNA was extracted according to manufacturer's protocol for the extraction of total RNA from yeasts with the following modifications for extraction from gram positive bacteria. *S. aureus* cells were lysed with 100 µl cell lysis buffer (1 M sorbitol, 0.1 M EDTA, 0.1% *β*-mercaptoethanol, 85 U Lysostaphin) incubated at 37°C for 20 min. Proteins were than denatured by adding 350 µl buffer RLT which contains a strong protein denaturant guanidine thiocyanate and mixed with a vortex mixture. A 250 µl volume of ethanol was added to create the conditions for the RNA to bind to the RNeasy® membrane, and applied to the RNeasy® spin columns. The RNA was washed to remove any contaminants using buffer RW1. An On-column DNase digestion was performed using the RNase–Free DNase set (Qiagen) to digest any DNA present on the membrane.

The DNA was digested by adding 80 µl of DNase I incubation mix (10 µl DNase I, 70 µl buffer RDD) directly to the RNease spin column and incubated at room temperature for 15 min. After incubation, the spin column was washed with buffer RW1 to remove the DNase I. The spin column was then washed twice with buffer

RPE and the RNA eluded with nuclease free water. RNA concentration was then measured with a Nanodrop and visualized with formaldehyde gel electrophoresis. RNA was stored at -80°C.

4.2.3. Formaldehyde Gel Electrophoresis

Agarose dissolved in NBC buffer (50 mM boric acid, 1 mM sodium citrate, 5 mM and 5 mM NaOH; pH 7.5) was incubated in a 65°C waterbath for 5 min and 2 ml formaldehyde was added. The gel was pre-run at 87 V for 20 min prior to evaluating the RNA sample. The RNA sample was prepared by adding 10 µl formamide, 3 µl 37% formaldehyde and 2 µl 10 × NBC buffer to 5 µl RNA and incubated at 65°C for 5 min. Loading dye and ethidium bromide was added to the sample to visualize on the formaldehyde gel.

4.2.4. Preparation of cDNA

cDNA was synthesized using the high capacity RNA to cDNA kit (Applied Biosystems) according to the manufacturer's protocol. A reverse transcription reaction was performed on about 1 µg total RNA, measured using a Nanodrop. One micro litre of triplicate RNA samples were pooled and the reverse transcription reaction was performed on about 1 µg of pooled RNA in a 20 µl final reaction mix containing 10 µl RT buffer (2x), 1 µl enzyme mix (20x) and nuclease free water up to a total of 20 µl. RT buffer contains both random hexamers and oligo dT primers and the enzyme mix contains the reverse transcriptase enzyme The reaction mix was incubated at 37°C for 60 min and the reaction was inactivated at 95°C for 5 min and cooled at 4°C. The intact cDNA was stored at -20°C.

4.2.5. Real-Time PCR

4.2.5.1. Qualitative real time PCR

Quantitative real time PCR was performed to screen for the presence of the *smr, qacG, qacJ* and *qacH* gene in *S. aureus* strains: VB1_qacG, VB2_qacH, VB3_qacJ, VB4_qacH and ATCC 25923 as well as the Avian pathogenic *Escherichia coli* (APEC) strain reported in Chapter 2. Real time PCR was performed on a LightCycler 2.0 instrument (Roche Diagnostics GmbH, Mannheim, Germany) with the *qac* primers which were described in Chapter 2 (Table 2.2). The optimized reaction mixture consisted of 5 µl cDNA, 2 µl primer mix (5 µm), 0.8 µl $MgCl_2$ (1.5 mM), 4 µl master mix (LightCycler® FastStart DNA Master^{plus} SYBR green 1, Roche

Diagnostics) and water up to 20 µl. The real time PCR run consisted of Taq activation at 95°C for 10 min followed by 45 amplification cycles (10 sec at 95°C, 10 sec at 57°C and 10 sec at 72°C). PCR reactions for each gene per strain were performed in separate reaction capillaries. SYBR green fluorescence emission was measured and continuously monitored during the run. Melting curve analysis was performed starting with a denaturation step by raising the temperature to 95°C, immediately followed by a drop to 65°C for 60 sec at a ramp rate of 20°C/sec and then raising the temperature to 95°C at a ramp rate of 0.1°C/sec, with continuous monitoring of the fluorescence. The temperature was then lowered to 40°C at a ramp rate of 20°C/sec and held at this temperature for 30 sec allowing for the single stranded PCR product to anneal and form double stranded PCR product.

4.2.5.2. Quantitative real time PCR

The following genes were used as Housekeeping (HK) genes during relative quantification: Pyrroline-s-carboxylate reductase gene (*proC*) that is involved in the amino acid biosynthesis, DNA gyrase A gene (*gyrA*) that is involved with replication and the transcription termination factor Rho gene (*rho*) that is involved with Transcription (Theis *et al*., 2007). The presence of the genes in all the strains were screened using the same procedure as described for qualitative real time PCR. The primer sequences and melting temperature are listed in Table 4.1. cDNA was prepared from the RNA extracted from strain VB4_smr control sample. The concentration of this sample was measured using a Nanodrop and this cDNA sample was selected as the calibrator. External standard curves of all three HK GENE (*proC, gyrA,* and *rho*) and *smr* (from here on end referred to as the gene of interest (GOI)) were constructed using triplicate tenfold dilutions of the calibrator sample starting with a concentration of 5 ng μl^{-1}. The standard curves were used to determine the PCR efficiency (E) of the different genes.

Quantitative real time PCR was performed to evaluate the expression of the *smr* gene in the QAC resistant *S. aureus* strains VB3_qacJ, VB4_smr and the QAC sensitive *S. aureus* strain ATCC 25923 cultivated in DDAC concentrations of 0.2 µg ml^{-1}, 1 µg ml^{-1} as well as a control of each strain cultivated with no DDAC added. Real-time PCR was run on a LightCycler 2.0 instrument (Roche Diagnostics GmbH, Mannheim, Germany) with small multidrug resistance primers (TIB MOLBIOL, Germany, Table 4.1). The optimized reaction mixture consisted of 5 µl cDNA, 2 µl primer mix (5 µm), 0.8 µl $MgCl_2$ (1.5 mM), 4 µl master mix (LightCycler® FastStart DNA Master^{plus} SYBR green 1, Roche Diagnostics) and water up to 20 µl.

Table 4.1. Primers used in the study for relative quantification

Gene	Primer	Sequence	Tm	Genome position
gyrA	gyrA A	5'-CTTGACCACTTGTTAAGCG-3'	51.8°C	9012-8994
gyrA	gyrA F	5'-CAATGATTGCTGTTAAAGACC-3'	51.0°C	8833-8853
proC	proC A	5'-TTTTCTAGACCAAGTTTCGTACC-3'	53.6°C	1619368-46
proC	proC F	5'-CAAACAACTTTGACCCTAAATCTA-3'	53.3°C	1619185-08
rho	rho A	5'-GGTCAACGTGGTTTAATAGTG-3'	51.7°C	2135638-18
rho	rho F	5'-TCTGTTACCTCTTCAGGACG-3'	51.9°C	2135496-15
smr	smr F	5'-ACGTTCATCATTCAACAGTCTA-3'	50.5°C	763-784
smr	smr A	5'-CAATGGCACGATATAAATAGC-3'	50.6°C	921-901

The real time PCR run consisted of Taq activation at 95°C for 10 min was followed by 45 amplification cycles (10 sec at 95°C, 10 sec at 57°C and 10 sec at 72°C). SYBR green fluorescence emission was measured and continuously monitored during the run. All samples were tested in duplicate. Melting curve analysis was performed as stated in section 4.2.5.1.

4.2.5.3. Expression of QAC resistance genes

S. aureus strains VB1_qacA, VB2_qacG, VB3_qacJ, VB4_smr, ATCC and Avian pathogen *Escherichia coli* (APEC) were cultivated in TSB for 16 h and used as an inoculum. Cells were inoculated to the OD of 0.1 and cultivated at 37°C. Triplicate samples were collected at 2 h, 4 h, 8 h and 22 h representing early exponential phase, mid exponential phase, early stationary and late stationary respectively. Cells were harvested by centrifugation for 5 min at 14 000 x *g* and 4°C. DNA was extracted from samples and stored at -20°C.

Strain VB4_smr was cultivated and samples collected in triplicate as described in methods above. RNA was extracted from the cells and cDNA synthesised as mentioned in section 4.2.4. The experiment was repeated with cells cultivated in TSB containing 0.2 µg ml^{-1} and 1 µg ml^{-1} DDAC.

4.3. Results

4.3.1. RNA isolation from *S. aureus*

Two methods of RNA extraction were compared for the use thereof in Real Time PCR, extraction using the RNeasy® mini-kit (Qiagen) and manual extraction with Tri-reagent. RNA concentrations as high as 824 ng $µl^{-1}$ were achieved with a 260/280 ratio of 2.16 using the RNeasy® mini-kit compared to 124 ng $µl^{-1}$ and 260/280 ratio of 1.94 achieved with the manual extraction. In Figure 4.1 formaldehyde gel electrophoresis profiles of RNA extracted are illustrated. The 23S rRNA and the 16S rRNA bands can clearly be visualised. The 23S rRNA and 16S rRNA clearly visible with distinct bands (Figure 4.1A) compared to the faint bands in Figure 4.1B. RNA extracted using the RNeasy® mini-kit in conjunction with the enzyme lysostaphin was the best method for the extraction of RNA from *Staphylococcus aureus* based on the high quality and quantity RNA and the clear visualised banding patterns (Figure 4.1A). RNA was subsequently extracted from *S. aureus* strains cultivated in DDAC concentrations of 0.2 µg ml^{-1} and 1 µg ml^{-1} as well as no DDAC as a control using the RNeasy® mini-kit. RNA concentrations as high as 935 ng µl and 260/280 ratio of 2.26 were obtained. Using RNA of the highest qualtity for real-time PCR and especially quantitative real time PCR is always advisable even though this technique can amplify low quality RNA. RNA from triplicate RNA extractions were pooled and 1 µg of RNA was transcribed to cDNA for real-time PCR.

4.3.2. Qualitative real time PCR

In Chapter 2 it was indicated that only one *qac* gene, corresponding to the strains name, could be amplified in the QAC resistant strains, VB1_qacG, VB2_qacH, VB3_qacJ, VB4_smr and VB5_qacA irrespective of cultivation in the presence or absence of QAC. None of the *qac* resistance genes could be amplified in the strain ATCC 25923 using conventional PCR but two of the *qac* resistance genes, *qacG* and *smr* were amplified in the APEC strain.

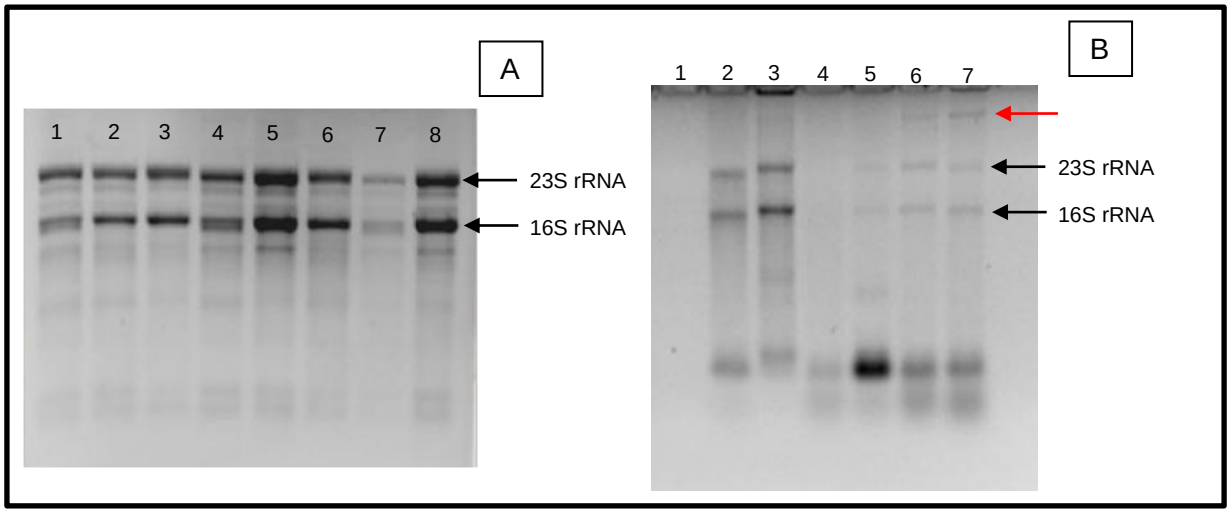

Figure 4.1. Formaldehyde gels visualised under UV illumination. A: Total RNA extracted from *S. aureus* strains cultured in TSB containing different concentrations of DDAC for 12 h using the RNease® mini-kit. Lanes 1-3 strain VB4_smr with no DDAC, 0.2 µg ml^{-1} and 1 µg ml^{-1} respectively. Lanes 4-6 strain VB3_qacJ with no DDAC, 0.2 µg ml^{-1} and 1 µg ml^{-1} respectively. Lanes 7-8 strain ATCC 25923 with no DDAC and 0.2 µg ml^{-1}, respectively. B: Total RNA extracted from *S. aureus* strains cultured in TSB for 12 h using manual tri-reagent method. Lane 1 strain ATCC 25923, lane 2 strain VB1_qacG, lane 3 VB4_smr, lane 4 VB5, lane 5 APEC, lane 6 VB3_qacJ and lane 7 VB2_qacH. Red arrow indicates DNA contamination and the black arrows indicate the 23 S rRNA and the 16SrRNA bands.

Using real time PCR, more than one *qac* resistance gene was amplified in all the *S. aureus* strains, including ATCC 25923 and APEC, where previously no genes could be amplified. Table 4.2 displays the results obtained from the LightCycler software. Listed are the CT values indicating product formation. The CT of the *smr* gene for strains ATCC 25923 was more than 40 as well as the *qacG* gene in the APEC strain. This was subsequently regarded as negative as the product could either not be visualised on an agarose gel or the size was not as expected thus unspecific amplification occurred (Figure 4.2). The strains VB3_qacJ, VB4_smr and ATCC 25923 were used in quantitative real time PCR where the expression of the *smr* gene was investigated. VB4_smr was used as a positive control because it was the strain with the lowest CT indicating more gene amplification, strain ATCC 25923 as a control strain because it was susceptible to QACs and strain VB3_qacJ because the *smr* gene could not be amplified in this strain using conventional PCR. The genes selected to be used as housekeeping (HK) genes were amplified in all the strains tested (Table 4.3). A HK gene should be able to be amplified in the strains used if it were to be used as HK in quantitative real time PCR assays.

Table 4.2. Real Time PCR results for the amplification of the *smr* gene and the housekeeping genes *rho, proC* and *gyrA* in the different *S. aureus* strains. Crossing point (C_T) is indicated as well as the melting temperature (T_m).

Strain	*smr*		*qacG*		*qacJ*		*qacH*	
	C_T	T_m	C_T	T_m	C_T	T_m	C_T	T_m
VB1_qacG	30.49	73.96	9.64	77.48	26.86	79.08	25.81	78.69
VB2_qacH	29.78	75.46	31.88	78.77	26.27	77.91	9.73	76.44
VB3_qacJ	28.22	73.27	35.43	78.36	9.21	77.79	29.57	80.67
VB4_smr	7.03	73.43	-	-	31.34	79.01	30.04	78.37
VB5_qacA	22.52	77.40	30.57	78.19	30.22	82.22	28.04	82.26
ATCC25923	>40.00*	76.63	37.29	78.39	27.56	74.19	27.98	78.01
APEC	-	-	>40*	71.16	34.17	85.08	29.54	74.90

*Crossing point (C_T) of 40 and more was regarded as negative. – Gene not amplified in the strain.

4.3.3. Relative quantitative real time PCR

4.3.3.1. SYBR green quantification

RNA was extracted from bacterial strains cultivated in media containing 0.2 µg ml^{-1}, 1 µg ml^{-1} and no DDAC in three separate cultivations. RNA to the concentration of 1 µg ml^{-1} was pooled from the three separate extractions and cDNA was synthesised from the pooled RNA samples and used in the relative quantitative real time PCR experiments. A cDNA sample synthesised from RNA extracted from cells grown without DDAC was selected as the calibrator. The calibrator sample is also referred to as the reference sample, i.e. the sample to which expression was normalised; a sample of known concentration and that contains the gene of interest (GOI), *smr*. This sample

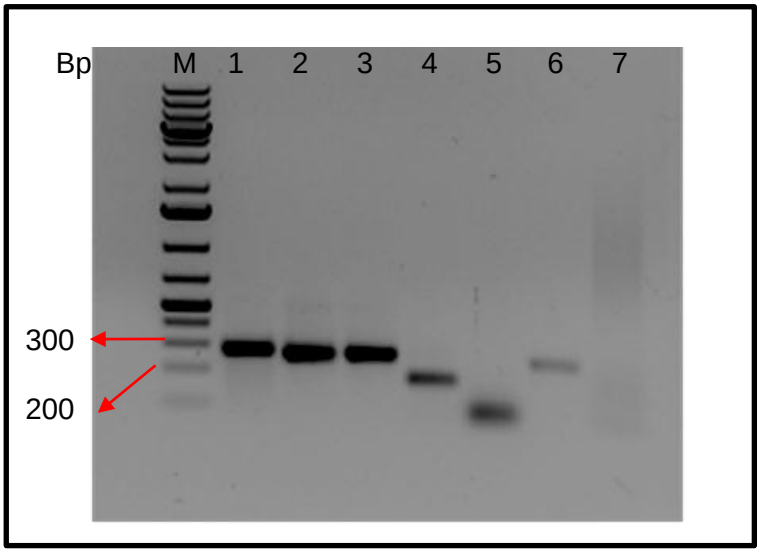

Figure 4.2. 1% w/v agarose gel visualised under UV illumination indicating QAC resistance genes amplified using real-time PCR. The gene products of *qacG* was 215 bp, *qacH* was 257 bp, *qacJ* was 216 and *smr* was 184 bp. Amplification of *qacH* with C_p of 9.73 (1), *qacG* with C_p of 9.64 (2), *qacJ* with C_p of 9.21 (3), *smr* with C_p of 7.03 (4), *smr* from ATCC strain with C_p of >40 (5), *qacG* from ATCC strain with C_p of 37.29 and the (6) and the amplification of the *qacG* from APEC with C_p of >40 (7)

Table 4.3. Real Time PCR results for the amplification of the housekeeping genes *rho, proC* and *gyrA* as well as the gene of interest, *smr* from the different *S. aureus* strains. Crossing point (CT) is indicated and the call target based on the Ct.

Strain	*proC*		*gyrA*		*rho*	
	CT	**T_m**	**CT**	**T_m**	**CT**	**T_m**
VB1_qacG	27.46	77.99	19.89	80.17	21.54	81.43
VB2_qacH	14.26	79.71	12.12	79.69	12.46	80.97
VB3_qacJ	23.63	80.33	19.74	79	18.31	81.78
VB4_smr	20.72	77.72	18.98	78.96	16.31	77.75
VB5_qacA	34	80.68	29.55	80.13	32	81.15
ATCC 25923	25.78	78.20	21.96	79.08	19.97	78.21
APEC	32	80.78	26.34	79.15	25.79	0.24

was used to generate standard curves for the housekeeping genes (HK) *gyrA, proC* and *rho* as well as the GOI, *smr*.

Relative expression ratios were calculated using the comparative ΔC_T method with PCR efficiency correction. Amplification efficiency is a measure of how efficient the gene concentration will double with every cycle in the PCR and was calculated from the slope of the standard curves. The PCR efficiency (E) for each gene was determined by generating triplicate cDNA dilution curves. The crossing point (Ct) for each cDNA sample was plotted against the log of the cDNA concentration. Due to the principle of calibrator normalized relative quantification exact copy number of standards is not necessary, relative dilution series of 1:10 was subsequently used. The slope of the standard curve (Figure 4.3) was determined and the PCR efficiency calculated according to the following formula: $E = 10^{(-\frac{1}{slope})}$ using the Roche LightCycler software.

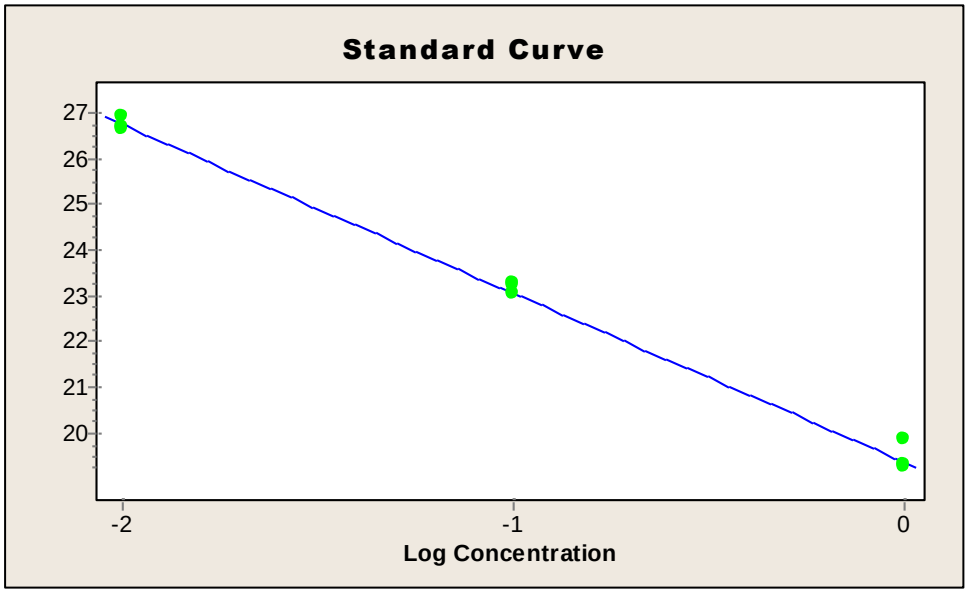

Figure 4.3. Standard curves calculated from the C_p of triplicate dilutions of the calibrator cDNA for the different housekeeping gene *gyrA* with an efficiency of 1.899.

Melting curve analysis was performed on the triplicate repeat samples used to generate the standard curve for the *gyrA* gene (Figure 4.4). This plot indicated that cDNA dilutions of 10^{-3} and higher indicated in a slight shift in the melting peaks and the subsequent cDNA dilutions the melting peaks also displayed a slight shift. Melting curve analysis was performed to identify any unspecific amplification. The high

dilutions displayed a slight shift in the melting temperature, but the shift in the melting temperature was considered as insignificant as the difference between the dilutions was under 1°C (Appendix II). The melting temperatures for the HK genes *gyrA, proC* and *rho* were 81°C, 80°C and 82°C, respectively and 79°C for *smr*. The fluorescence decreased at the higher dilutions. This was expected as lower concentrations for the products were expected at these low cDNA concentrations.

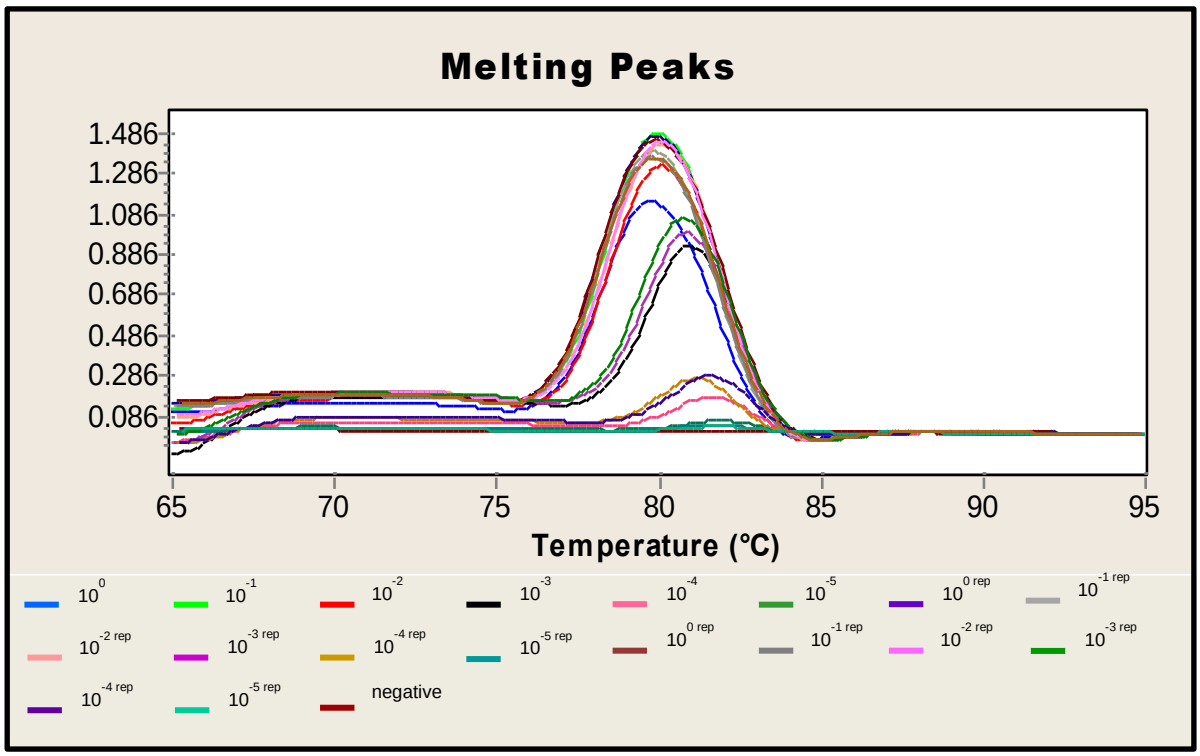

Figure 4.4. Melting peak analysis of *gyrA* gene after the relative quantification using SYBR green indicating that the primers were specific and no secondary products were amplified.

The LightCycler software disregarded these low concentrations during the generation of the standard curve in the determination of the PCR efficiency, E (Figure 4.3; Appendix II). Positive amplification was still achieved at the low concentrations (CT Appendix II). The same trend was observed for the standard curve generation of all the HK genes (Figure 4.5 – 4.8) as well as for the GOI, *smr* (Figure 4.9 – 4.10).

Normalization of the expressed gene ratio was achieved using the geNorm software (Vandesomple *et al*., 2002; http://medgen.ugent.be/genorm/). This software takes multiple housekeeping genes into account during the normalization of the gene

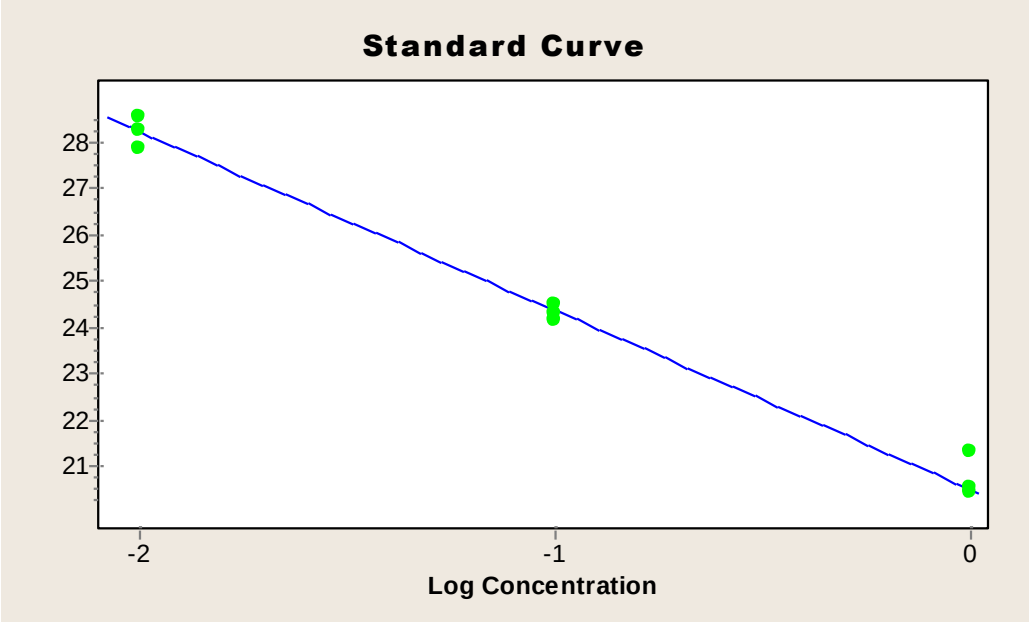

Figure 4.5. Standard curves calculated from the Ct of triplicate dilutions of the calibrator cDNA for the different housekeeping gene *proC* with an efficiency of 1.967.

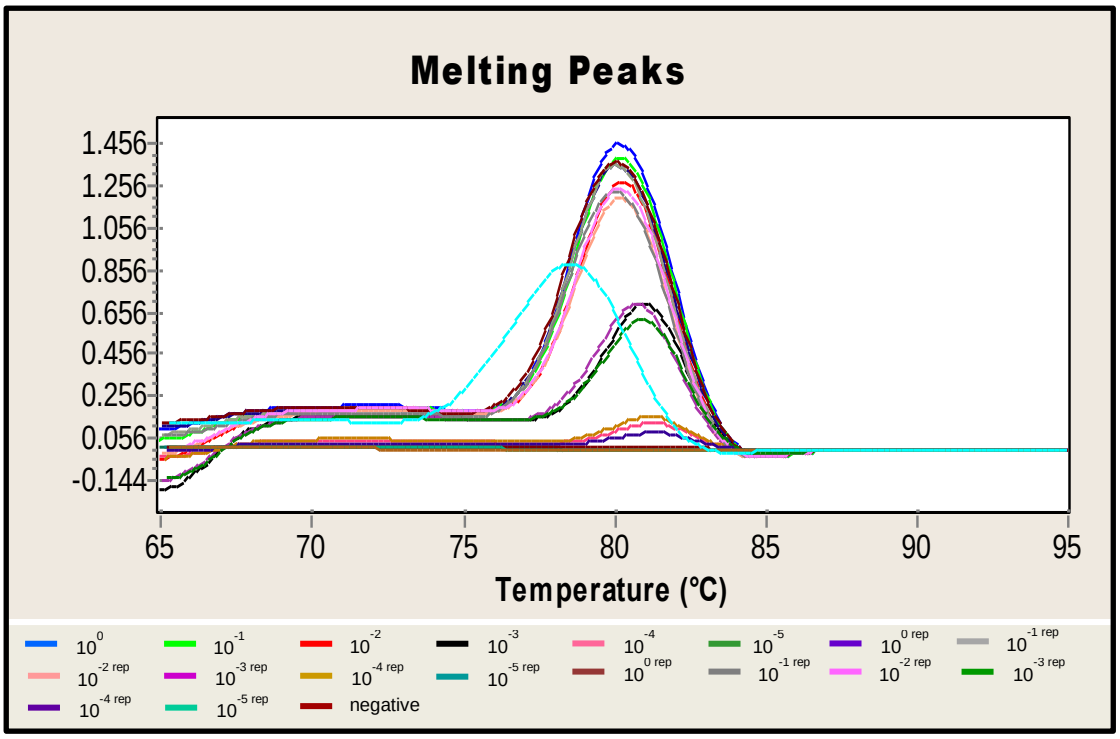

Figure 4.6. Melting peak analysis of *proC* gene after the relative quantification using SYBR green indicating that the primers were specific and no secondary products were amplified.

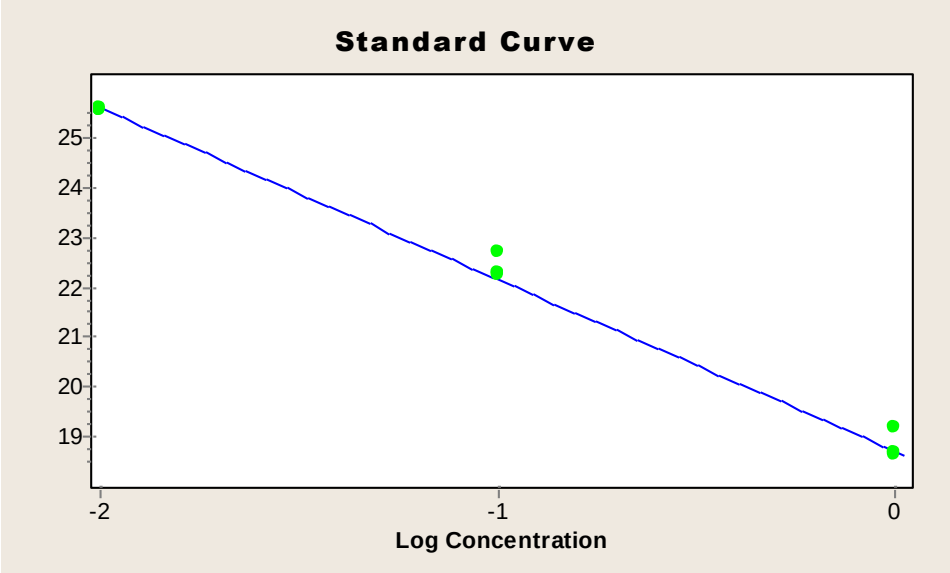

Figure 4.7. Standard curves calculated from the Ct of triplicate dilutions of the calibrator cDNA for the different housekeeping gene *rho* with an efficiency of 1.817.

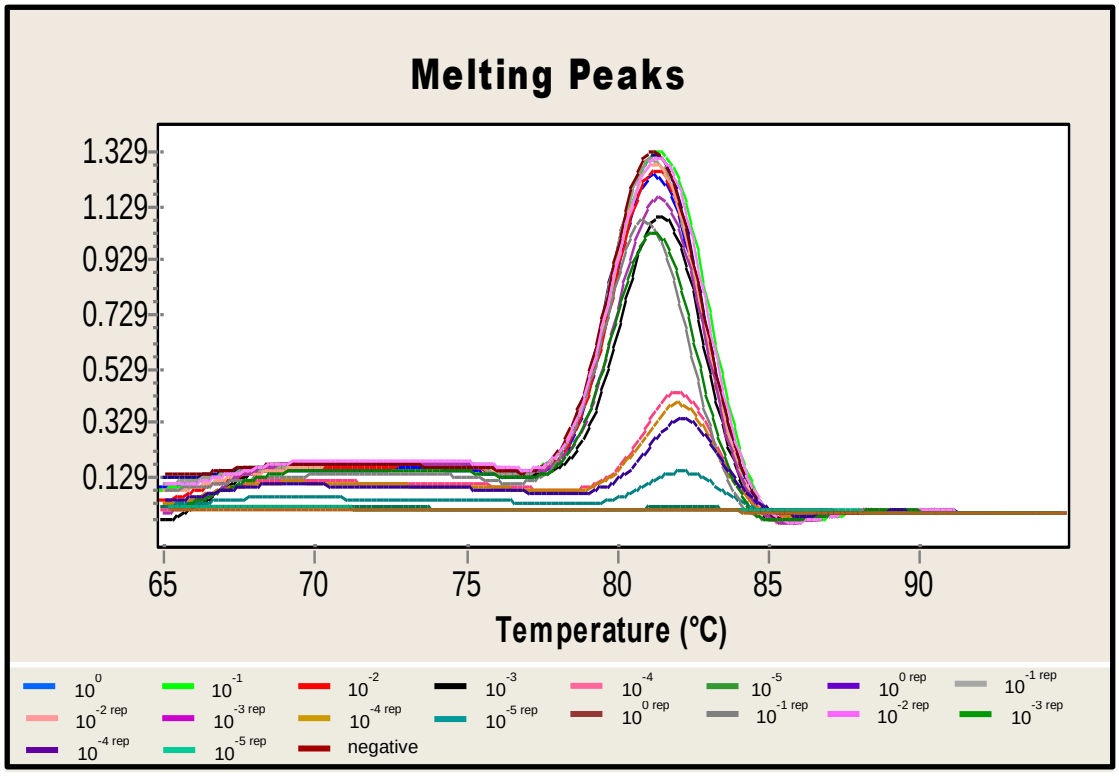

Figure 4.8. Melting peak analysis of *rho* gene after the relative quantification using SYBR green indicating that the primers were specific and no secondary products were amplified.

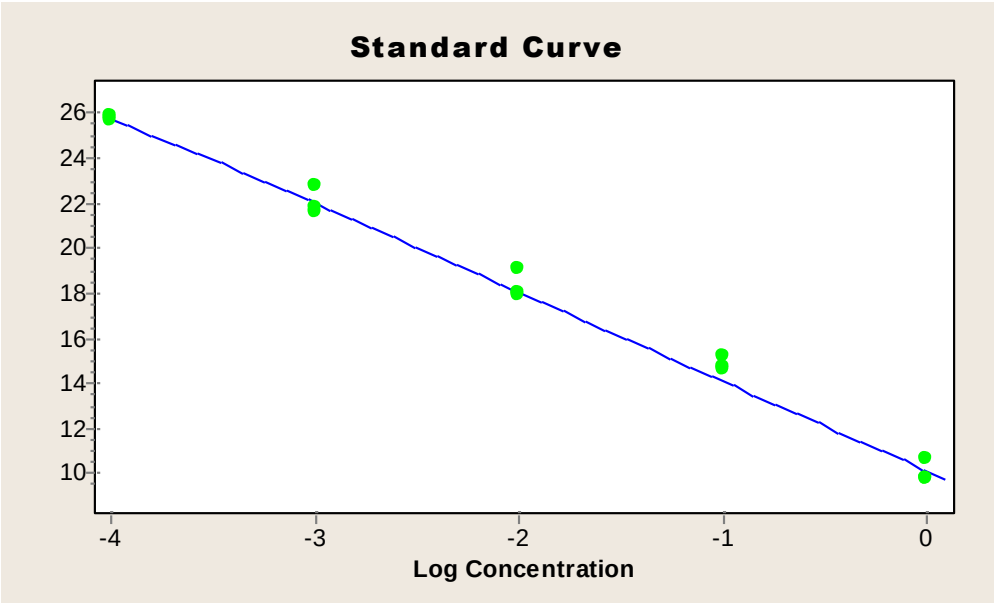

Figure 4.9. Standard curves calculated from the Ct of triplicate dilutions of the calibrator cDNA for the different housekeeping gene *smr* with an efficiency of 1.7.

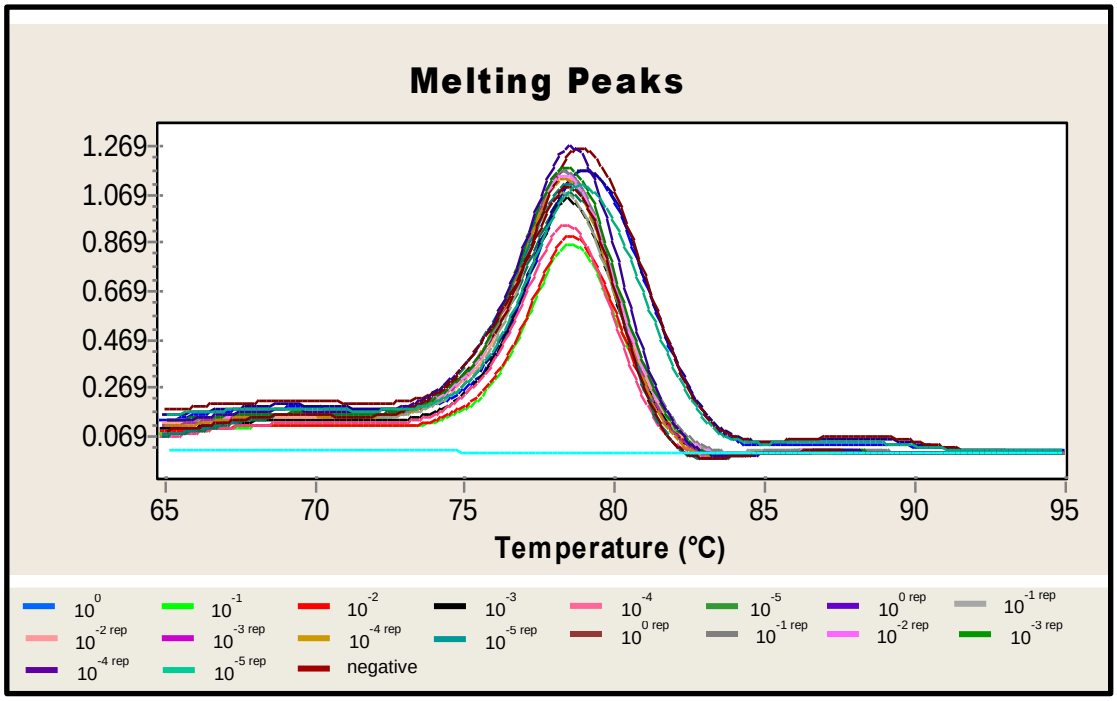

Figure 4.10. Melting peak analysis of *smr* gene after the relative quantification using SYBR green indicating that the primers were specific and no secondary products were amplified.

expression ratios. The CT values obtained from the LightCycler software was transformed into quantities using the formula $Q = E^{(calibrator\ C_P - sample\ C_P)}$ where Q is the quantities and E the PCR efficiency. The quantities of the GOI were then divided by the geometric mean of the Housekeeping gene quantities to normalize the quantities of the GOI against the HK genes.

For relative quantification a calibrator was used; a sample of known concentration containing the GOI, *smr*. The calibrator in this case was cDNA synthesised from RNA extracted from the strain VB4_smr. The quantities of all the HK genes (*gyrA, rho* and *proC*) were used in the calculation to compensate for slight differences in the sampling, extraction of RNA or the synthesis of cDNA. The relative amount of induced *smr* was plotted (Figure 4.11). Under conditions in the absence of DDAC *smr* was expressed for the strain VB4_smr but not for VB3__qacJ or ATCC. The expression of the *smr* gene induced in the presence of 0.2 µg ml^{-1} DDAC where a twofold increase in the expression of *smr* was observed for the VB4_smr with

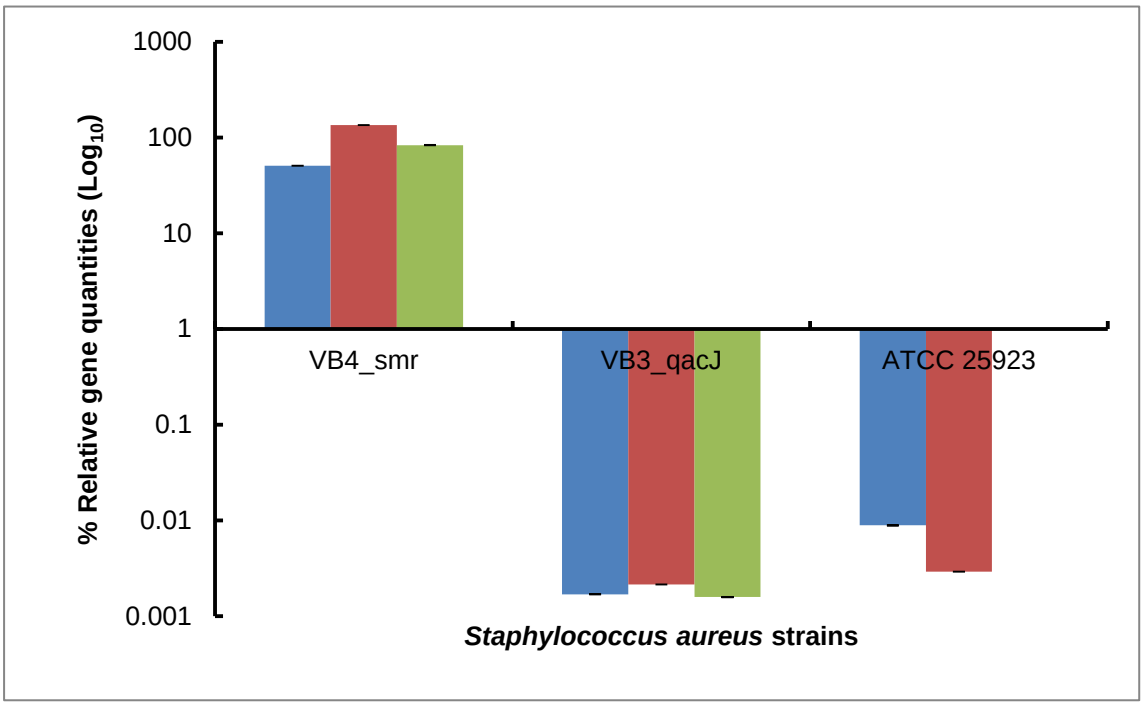

Figure 4.11. Relative amounts of *smr* gene expression in *S. aureus* strains grown without DDAC ■, 0.2 µg ml^{-1} DDAC ■ and 1 µg ml^{-1} DDAC ■. Strains VB4_smr and VB3_qacJ are representatives of QAC resistant *S. aureus* strains containing *smr* gene and *qacJ* gene respectively. Strain ATCC 25923 is susceptible to QACs. Error bars represent standard deviation of 2 technical repeats.

standard error between the data points (Table 4.4). Induction of *smr* was also observed in the presence of 1 µg ml^{-1} DDAC but the induction of the gene was lower than in the case of induction with 0.2 µg ml^{-1} DDAC. The *smr* gene was not induced or expressed in the strains VB3_qacJ or ATCC.

Table 4.4. Normalised gene quantities for *smr* and error calculated using geNorm software

Organisms	No QAC		0.2 µg ml^{-1} DDAC		1 µg ml^{-1} DDAC	
	Quantity	**Error**	**Quantity**	**Error**	**Quantity**	**Error**
VB4_smr	5.09E-01	1.08E-01	1.36E+00	2.19E-01	8.35E-01	3.80E-01
VB3_qacJ	1.70E-05	6.44E-06	2.15E-05	2.12E-06	1.59E-05	3.76E-06
ATCC 25923	8.89E-05	4.19E-05	2.92E-05	4.80E-06	NA	NA

ANOVA single factor analysis was used to test for difference in the expression level of the *smr* gene in bacterial cells cultivated in different concentrations of DDAC with a level of significance of $P \leq 0.05$ (Appendix II). There was no difference in the expression levels of the *smr* gene given that $p > 0.05$. Melting curve analysis was performed on the gene products after relative quantification. The Tm for all the PCR products was about 75°C indicating that the PCR products were *smr* and not unspecific amplification (CT values with Tm, Appendix II).

Expression of *smr* was investigated over a 22 h time interval according to the different growth stages of *S. aureus*, early exponential, late exponential, early stationary and late stationary phase (Figure 4.12). Expression of *smr* was compared in cells grown without DDAC and with 0.2 µg ml^{-1} DDAC. RNA was extracted from cells harvested at 2h, 4h, 8h and 22h, the time intervals representing the different growth stages of *S. aureus*. The relative expression values with error values calculated using the geNorm software (Table 4.5).

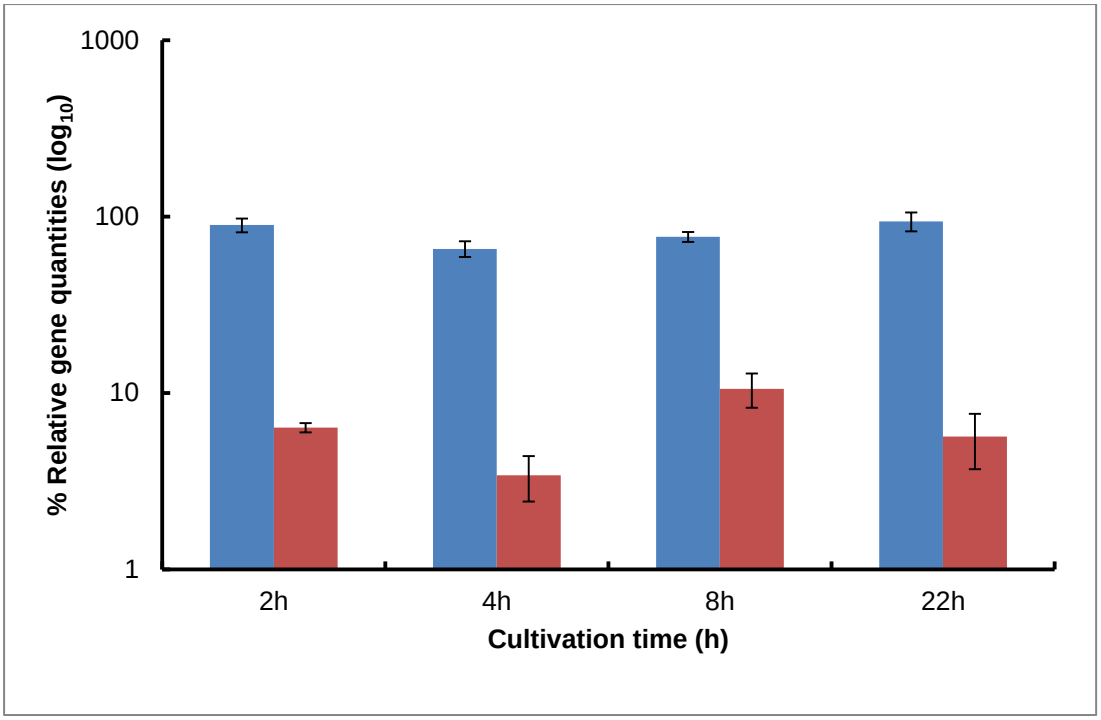

Figure 4.12. Relative amounts of *smr* gene expression in *S. aureus* strains cultivated in media containing no DDAC ■ and 0.2 µg ml^{-1} DDAC ■ for 22 h. Error bars represent standard deviation of 2 technical repeats.

Table 4.5. Normalised gene quantities for *smr* and error calculated using geNorm software

Time	No QAC		0.2 µg ml^{-1} DDAC	
	Quantity	**Error**	**Quantity**	**Error**
2 h	8.97E-01	8.06E-02	6.37E-02	3.95E-03
4 h	6.57E-01	6.70E-02	3.42E-02	9.85E-03
8 h	7.70E-01	4.94E-02	1.06E-01	2.33E-02
22 h	9.41E-01	1.14E-01	5.66E-02	1.96E-02

ANOVA two factor analysis were performed on the relative expression quantities of *smr* to test for the difference in the expression level of the *smr* gene in bacterial cells

cultivated in different concentrations of DDAC and different time intervals (Appendix II). There was a significant difference between the different cultivation conditions, with $p<0.05$, but there was no significant difference in the expression of *smr* gene over a time interval regardless of the QAC treatment with $p>0.05$. The interaction between the different QAC treatments and time interval revealed no significant difference since $p>0.05$. This could clearly be seen in Figure 4.12 where the relative quantities were lower in the case of the DDAC treatment contrary to what was found in the above mentioned study where DDAC treatment induced the expression of the *smr* gene (Figure 4.11). The CT values and melting temperature for the expression studies are found in Appendix II.

4.4. Discussion

Bacteria have the ability to develop resistance to a wide range of harmful environmental conditions and often resistance is conferred through the presence of resistance genes. Generally these genes are associated with class 1 integrons that are usually found on resistance plasmids and have mostly been found in clinical environments. Recently it was shown that the QAC resistance genes are also associated with class 1 integrons that are widely spread in the natural environment and not only in a clinical environment (Gaze *et al*., 2005; Gillings *et al*., 2008 a; b; 2009a & b). Integrons are mobile genetic elements that usually contain gene cassettes and transposons that contain the resistance genes. These genetic elements ensure the transfer of resistance between species.

The *qac* resistance genes code for small protein pumps that do not need the cooperative function of any other gene to function where one gene codes for a fully functioning protein pump (Kumar & Schweizer, 2005).This chapter focused on amplifying the different *qac* resistance genes using real time PCR a technique more sensitive than conventional PCR. The hypothesis that bacteria possess all the different *qac* resistance genes was investigated by amplifying the different *qac* genes *qacG, qacH, qacJ* and *smr* from DNA extracted from the bacterial strains cultivated in a medium with no QAC added. Previously in Chapter 2 it was discussed that only one specific gene was amplified in the bacterial strains resistant to QACs. A susceptible strain, ATCC 25923 was included in the study as a control strain and none of the *qac* genes was amplified in this particular strain using conventional PCR.

The different *qac* genes, *qacG, qacH, qacJ* and *smr* were amplified using real time PCR using the same PCR conditions and primers as in the conventional PCR

mentioned in Chapter 2. Real time PCR was more sensitive than the conventional PCR since it was able to amplify genes that previously in Chapter 2 could not be amplified. This study thus confirmed the study done by Gillings and co - workers (2009 a; b) where the *qac* resistance genes were amplified in environmental bacterial species where QACs are not part of the ecosystem. During this study, the *qac* resistance genes used were amplified in most of the resistant strains including amplification of the *qac* resistance genes from the control strain, ATCC 25923 (Table 4.3). This indicates that more than one SMR efflux genes could be present in the resistant strains. This opens the door to more questions as to why only certain genes were amplified with conventional PCR and this prompted the investigation into the expression of these genes when induced with QACs.

A second hypothesis was formulated and this was that the *qac* genes were induced in the presence of QAC and thus the expression should be increased in the presence of a QAC. In order to perform expression studies real time PCR was used again as this is the technique mostly used for expression studies today because of its sensitivity and speed. Previously the presence of the QAC resistance genes have been identified with the use of the northern blot hybridization and chemi luminescence techniques (Heir *et al*., 1998; 1999a & b; Bjorland *et al*., 2005). The LightCycler uses glass capillaries as reaction vessels and a reaction chamber in which the air temperature is rapidly controlled by a microprocessor driving a fan and heating a lamp permitting rapid temperature cycling during PCR (Stöcher *et al.,* 2002). After SYBR green amplification, products are analysed by measuring the fluorescence emitted by the fluorescent dye SYBR green, thus the PCR products are amplified and detected in the same step. Positive amplification of a gene depends on the crossing point (CT) of the cycles and a CT 40 was decided as being a negative. This was illustrated in the agarose gel photo (Figure 4.2) where a gene product of CT 40 gave unspecific amplification or no product.

The method of gene quantification chosen for this study was the relative quantification method with calibrator controls. This method was chosen because there is no need for precise quantities of cDNA and it allows for any variations that could have slipped in at any point during the experiment such as cell harvesting, RNA extraction and cDNA synthesis. Normalization of gene expression data is used to correct sample-to-sample variation (Ririe *et al*., 1997; Vandesomple *et al*., 2002). Starting material obtained from different individuals usually varies in tissue mass or cell number, RNA integrity or quantity or experimental treatment and the effect of the DDAC on the resistance strains was not known thus normalization was necessary.

The calibrator that was incorporated into this method was a cDNA sample of which the concentration was known and that contains the gene of interest (GOI), *smr*. The comparative C_T method ($2^{-\Delta\Delta CT}$) method for quantification was used to normalise the expression of the genes. This method includes the use of housekeeping genes to which the expression of the *smr* gene is normalised, thus the expression of the GOI was given as a ratio.

The Pfaffl model combines gene quantification and normalization into a single calculation incorporating the amplification efficiencies of the target and reference (housekeeping) genes to correct for differences between the two assays (Pfaffl *et al.,* 2002). Real-time PCR results were normalized against a control gene the calibrator. Theis and co-workers (2007) investigated a range of genes that could be used as housekeeping genes specifically in *Staphylococcus aureus* gene quantification assays. Genes used as housekeeping genes are specifically selected as they must not be influenced by the experimental conditions and should be present in all the organisms used in the study. The genes chosen in this study was the Pyrroline-s-carboxylate reductase gene (*proC*) that is involved in the amino acid biosynthesis, DNA gyrase A gene (*gyrA*) that is involved with replication and the transcription termination factor Rho gene (*rho*) that is involved with Transcription (Theis *et al*., 2007). The housekeeping genes were positively amplified in all the bacterial strains tested (Table 4.3) and the melting temperature of the different genes did not differ by more than 4°C. There were no differences in the genes compared between the strains.

Using the comparative C_T method ($2^{-\Delta\Delta CT}$) method, PCR efficiency (E) was incorporated into the calculation. PCR efficiency is a measure of how efficient the PCR was. Efficiency was calculated from the slope of the standard curve, thus for each of the HK genes and the GOI a standard curve analysis was performed using the calibrator sample (Figure 4.3- 4.6). The LightCycler software allowed for the use of external controls thus it was not needed to incorporate a standard curve with each run, but when reagents or kits was changed, a new standard curve had to be set up again. It was also very important to use the same calibrator for the entire experiment as this was included in each run to normalize the gene quantities.

All three HK genes, *rho, proC* and *gyrA* were used to normalize the gene expression in the *smr* genes using geNorm software (Vandesomple *et al.,* 2002). The LightCycler software only allows for the incorporation of one HK gene and because in practice there was differences in the expression of the HK genes all three of the HK

genes was incorporated. Smith and co-workers (2008) found that with the increase in disinfectant concentration, the expression of the gene increased. They looked at the expression of a repressor gene, *qacR* that regulates the expression of the *qacA/B* gene in the presence of QAC. The *qacA/B* genes codes for the proteins QacA and QacB that has been shown to be part of the major facilitator superfamily of multidrug transporters and not part of the SMR transporters (Gaze *et al*., 2005; Kumar & Schweizer, 2005).

Induction of the *smr* gene was investigated in the *S. aureus* strains VB3_qacJ, VB4-smr and ATCC 25923. Strain VB4-smr was used as a positive control as this strain was the only strain in which the *smr* gene was previously amplified using conventional PCR. Strain VB3-qacJ was chosen as a representative of a QAC resistant bacterial strain and this strain was the only strain in which the *qacJ* gene was amplified using conventional PCR and the *smr* gene was amplified in this strain with real time PCR from DNA extracted from this strain. The ATCC strain was selected as a control strain as this strain was susceptible to QACs and this was the only strain where none of the genes were amplified using conventional PCR. The *smr* gene was present in high concentration in the strain VB4-smr during control conditions i.e. conditions where no DDAC was present and not in the strains ATCC and VB3_qacJ (Figure 4.11). This corresponded to the findings in Chapter 2 where the strain VB4-smr was the only strain where the *smr* gene could be amplified using conventional PCR.

The addition of 0.2 µg ml^{-1} DDAC induced the expression of the *smr* gene and the relative quantities had a more than two-fold increase in the expression of the gene (Table 4.4), but ANOVA analysis revealed that the difference in the expression was not significant. This was also observed for the induction with 1 µg ml^{-1} DDAC but the gene concentration was lower than in the case of induction with 0.2 µg ml^{-1} DDAC. There was no induction of the genes in the strains ATCC 25923 or VB3_qacJ. The strain VB3-qacJ grew in 0.2 µg ml^{-1} and 1 µg ml^{-1} DDAC thus another mechanism might be involved that made it possible for this strain to grow in this high concentration of DDAC. In Chapter 2 it was indicated that if a strain was deemed resistant it was able to grow at 1 µg ml^{-1} DDAC below its MIC for DDAC. ATCC 25923 was unable to grow at 1 µg ml^{-1} DDAC. It was expected for the ATCC strain that no induction of the genes were present as this organism is susceptible to QACs.

Induction of the *smr* gene was observed after a 16 hour incubation period in DDAC and it was thus prudent to look at the expression of the *smr* gene. The expression of

the gene was investigated and RNA was extracted at 2 h, 4 h, 8 h and 22 h which roughly represented the different growth stages of *S. aureus* which was early and late exponential as well as early and late stationary. RNA was extracted at roughly the same time points for both control conditions where no DDAC was present and 0.2 µg ml^{-1} DDAC. Here a completely different picture was observed for the strain VB4-smr (Figure 4.12). There was a tenfold decrease in the expression of the *smr* gene in the case of DDAC treatment compared no DDAC treatment (Table 4.5). A slight fluctuation in the expression of the smr gene between the time intervals for both the DDAC and non-DDAC treatments was observed. Two factor ANOVA analysis revealed that the difference between the time intervals was not significant, but there was a significant difference in expression of *smr* gene between the different DDAC treatments.

Bacteria may contain an array of different multidrug transport proteins that enable them to respond to a wide array of environmental signals (Putman *et al*., 2000). This seems to be true in the case of the SMR proteins and could account for the decrease in expression for the *smr* gene upon increase in the DDAC concentration in the cultivation media where a different gene in the SMR family might be induced or possibly any of the multidrug resistant family of transport proteins causing the *smr* gene's expression to decrease. The fluctuating levels of the genes could possibly also indicate that the cellular functions could possibly be used for maintenance and because the cells are in a stressful environment then energy of the cell could be alternating into functions to repair damage and pumping out of the toxic compound.

Huet and co-workers (2008) suggested that there might be a limited amount of multidrug proteins tolerated by the bacterial system and the excess transcripts could possibly be degraded. Translation of the mRNA might be blocked at the ribosomes and thus the expression level of the gene might not correlate to the protein that is expressed (Grundy & Henkin, 2006). The fluctuations observed could also possibly be explained by the promoter for the *smr* gene. Kierzek and co-workers (2001) showed that the promoter does have a significant effect on the expression of genes because a weak promoter expresses the proteins in "bursts" that occur at random intervals, but in a comparison between the promoter of the *smr* and *qacG* gene Heir and co-workers (1999) found that *smr* had a stronger promoter than *qacG*. It would be prudent to look at the expression at protein level as well to conclude this study.

The *qac* resistance genes form part of a *qac* gene cluster (Gillings *et al.,* 2009 a; b) and there is a possibility that they function as an operon. This is usually the case in

bacteria where groups of proteins work together to execute a function (McAdams & Shapiro, 2003). The different multidrug proteins could be working together where some are activated while others are down regulated. Thus it is very important to look at the expressions of the different proteins as a whole to see the clear picture of the mechanisms involved in resistance. After this work, it is apparent that resistance to QAC is not straight forward, and is not simply dependent on the presence or absence of a particular *smr* gene, or combinations of these genes. QAC resistance is also not dependent on elevated expression levels of a particular *smr* gene when the bacterium is grown in the presence of a QAC. QAC resistance thus appears to be an extremely complex situation which will require much further study.

Chapter 5
General Discussions and Conclusions

Quaternary ammonium compound (QAC) resistance has become a major concern since the discovery of resistance in food associated bacterial strains. Resistance is associated with the presence of the *qacG, qacH, qacJ* and *smr* genes that code for the small multidrug resistance pumps (Paulsen *et al.,* 1995; Heir *et al.,* 1998; 1999; Bjorland *et al.,* 2003). The small multidrug resistance genes also named the *qac* resistance genes are one of the smallest transporters known that actively transport toxins and disinfectants out of the cell (Schuldiner *et al.,* 1997; Borges-Walmsley & Walmsley, 2001).

During this study, four QAC resistant *Staphylococcus aureus* strains were obtained as well as a *S. aureus* strain which is susceptible to QAC (strain ATCC 25923). The minimal inhibitory concentration (MIC) of these strains were determined for the different QACs, namely benzalkonium chloride (BC), alkyldimethylammonium chloride (AAC), didecyldimethylammonium chloride (DDAC) and the commercial product called Virukill. Virukill was selected as a representative of a commercially available QAC. DDAC is also the active ingredient of Virukill, BC was selected as a representative of a first generation QAC and AAC as a second generation QAC and this is also the active ingredient in many commercial QACs based products.

It has been hypothesized that detergents, and in particular QAC based detergents, cause disorganization of the cell membrane leading to leakage of intracellular material and ultimately causes cell death (Salton, 1951). It was not until Yoshimatsu and Hiyama (2007) observed bleb formation on *Escherichia coli* cells after treatment with didecyldimethylammonium chloride that this was proven. Wattanaphansak and co-workers (2010) confirmed this observation when they observed the particle-like debris from bacterial membranes form on the cell membranes after treatment with disinfectants. During the current study, the same morphological changes were observed (Figure 3.1 and 3.3).

SEM nano-etching with an Argon gun with the added ability to perform elemental analysis using a nanoprobe was previously performed for the first time on biological samples (Swart *et al*., 2010). This technology has now been successfully applied on

bacterial cells (*S. aureus*) for the first time. Nano-etching was achieved by sputtering the sample with ionized argon atoms, thereby peeling 8.5 nm segments from the sample after every electron sputtering under a vacuum and high pressure. This enabled the detection of the 3-D nanostructure of cells and DDAC-treated cells. This study was the first study where the elemental composition of these 'blebs' or nodules was analysed. Elemental composition depth profiles of cells were obtained to make quantitative assessments of the elemental composition as etching proceeded (Figure 3.7). The depth profile method was capable of determining the elemental composition of the cells and revealed that there was no difference between the elemental composition of the cell membrane or the nodule, indicating that it was part of the cell membrane or cellular material and not the culture medium.

QACs are membrane active compounds and thus it would be expected that once etching reached the cell membranes, QAC would be detected. Only trace amounts of chlorine could be detected in the treated cells and none was detected in the control cells. This was expected as no DDAC was present in the control cells. As only trace amounts of chlorine were detected in the treated cells, this raises questions as to what happened to the QAC. Possible reasons for the disappearance of the chlorine could be that the chlorine is cleaved from the QAC molecule upon entering the cell membrane and was washed away during the steps in preparation for SEM or the QAC was modified by the cell and thus it could not be detected. The later could possibly be the reason as Gilbert and Moore (2005) suggested that the QACs form micelles with part of the cell membrane. The cell membrane encapsulates the QAC thus it could possibly evade detection. Yoshimatsu and Hiyama (2007) found that the bleb formation was only seen at higher concentrations of DDAC, but in this study (Figure 3.1) the nodule formation was seen at concentrations as low as 0.4 µg ml^{-1}. No nodules were seen on the control cells in the absence of DDAC (Figure 3.3 and 3.4), this indicates that the nodule formation is associated with the presence of DDAC but only at the higher concentrations did it affect the cells negatively.

Programmed cell death gives another possible reason for cell death where some unicellular organism have been shown to die in a way that resembles apoptosis, in this case cell necrosis, where biochemical events lead to unusual cell changes (Benndorf *et al*., 2004). These changes include blebbing, swelling, bursting and spilling cellular content (Raff, 1998). Bacterial apoptosis or the leakage of cellular material has been demonstrated in this study where empty cells were observed when cells were treated with DDAC (Figure 3.9). Bacterial apoptosis might be an important

factor that could cause the death of cells during antibacterial activity and it could also play an important role in the process that allows bacteria to become resistant to these substances. The cells themselves were damaged to the extent that when the high pressure and cellular bombardment from the electron gun was applied the cells completely collapsed, something that was not observed in the control cells (Figure 3.9).

The QAC genes, *smr, qacG, qacJ* and *qacH* were identified in the respective resistant strains (Figure 2.4 and Figure 2.5). The minimal inhibitory concentration (MIC) against the strains containing the QAC resistance genes confirmed the findings by Paulsen and co-workers (1995) that the presence of these genes confer resistance to QACs in these organisms. Strains were regarded as resistant to QACs when they could tolerate 2 µg ml^{-1} or more for any QAC. The strains containing *qac* resistance genes could tolerate high QAC concentrations.

The ATCC strain was susceptible to all of the different generations of QACs except for BC which had a MIC of 5.8 µg ml^{-1}. BC was one of the first QACs developed and it has been shown that most bacteria are resistant to this QAC and this was evident in the MIC for the control strain ATCC 25923 of 5.8 µg ml^{-1}. When the MIC was determined during a contact time of 20 min, the MICs was higher than compared to an MIC when the cells were exposed to QACs for a long time. The MICs of these bacteria could potentially be much higher in a setting outside the lab as they could grow in conditions such as biofilms that would make them much more resistant to biocides (Campanac *et al.,* 2002). There is no significant evidence that the different *qac* genes confer different levels of resistance to the different QACs (Table 2.7).

Bacteria have the ability to transfer resistance genes between species and thus increase the spread of these genes. The fact that these genes are found on mobile DNA elements also makes them very transient and thus the organism could lose them at any point in time resulting in the organisms becoming more susceptible to the toxic compound. Disinfection is an important factor to general hygiene and it could possibly minimize the use of antibiotics but knowledge on the correct use of these substances is vital to the continued successful use of these products. Recently it was shown that the QAC resistance genes can be found on class 1 integrons that is widely spread in the natural environment and not only in a clinical environment (Gaze *et* al., 2005; Gillings *et al*., 2008 a; b; 2009 a ; b).

Real time PCR was introduced in this study since it is a technique known to be more sensitive than conventional PCR. Because *qac* resistance genes are widely spread in nature it was proposed that the susceptible strain ATCC 25923 could potentially carry some of these genes, thus explaining the strains resistance to benzalkonium chloride (BC). The sensitivity of the real time PCR was used to amplify the different *qac* resistance genes in this strain and all the other strains that was potentially in too low concentrations for conventional PCR.

The real time PCR studies confirmed the study done by Gillings and co-workers (2009 a; b) where all the *qac* resistance genes were amplified in most of the resistant strains including amplification of the *qac* resistance genes from the control strain, ATCC 25923 (Table 4.3). This indicates that more than one SMR efflux genes could be present in the resistant strains. This opens the door to more questions as to why only certain genes were amplified with conventional PCR and this prompted the investigation into the expression of these genes when induced with QACs. Thus the presence of the *qac* genes seems to be not the only factor to render bacteria resistant to QACs.

In Chapter 2 the strain VB4-smr was the only strain where the *smr* gene could be amplified using conventional PCR. The addition of 0.2 µg ml^{-1} DDAC induced the expression of the *smr* gene where the relative quantities had a more than two-fold increase in the expression of the gene (Table 4.4). There was no induction of the genes in the strains ATCC 25923 or VB3_qacJ. The strain VB3-qacJ grew in 0.2 µg ml^{-1} and 1 µg ml^{-1} DDAC thus another mechanism might be involved that made it possible for this strain to grow in this high concentration of DDA findings in this study corresponded to Smith and co-workers (2008) findings were an increase in disinfectant concentration expression of the gene increased. They looked at the expression of a repressor gene, *qacR* that regulates the expression of the *qacA/B* gene in the presence of QAC. Induction of the *smr* gene was investigated in the *S. aureus* strains VB3_qacJ, VB4-smr and ATCC 25923. The *smr* gene was present in high concentration in the strain VB4-smr during control conditions i.e. conditions where no DDAC was present and not in the strains ATCC and VB3_qacJ (Figure 4.11).

Bacteria may contain an array of different multidrug transport proteins that enable them to respond to a wide array of environmental signals (Putman *et al*., 2000). This seems to be true in the case of the SMR proteins as well and could account for the decrease in expression for the *smr* gene upon increase in the DDAC concentration in

the cultivation media where another gene in the SMR family might be induced and the expression of the *smr* gene was decreased. Another possibility could also just be when the sampling DDAC treated cells, cells were not all at the same growth phase as DDAC concentration do affect the growth rate of the cells and the cells were not harvested in the correct growth phase. This could also be seen where the CT of the housekeeping genes increased for the DDAC treated cDNA. The fluctuating levels of the genes could possibly also indicate that the cellular functions could possibly be used for maintenance and because the cells are in a stressful environment then energy of the cell could be alternating into functions to repair damage and pumping out of the toxic compound.

The *qac* resistance genes form part of a *qac* gene cluster (Gillings *et al.,* 2009 a; b) and there is a possibility that they function as an operon. This is usually the case in bacteria where groups of proteins work together to execute a function (McAdams & Shapiro, 2003). The different multidrug proteins could be working together where some are activated while others are down regulated. Thus it is very important to look at the expressions of the different proteins as a whole to see the clear picture of the mechanisms involved in resistance.

To conclude, it was observed that there is slight threat to the development of QAC resistance in bacteria. Bacteria are able to cope with any unfavourable environment. Some studies indicated that when a certain antibiotic has not been in use, the organisms become susceptible to that particular antibiotic again. Thus it would be wise to alternate the different QACs used in a particular environment and use the correct disinfection programs. Resistance to QAC is not straight forward and combinations of different genes play a role in this process and will require further study.

Summary

The widespread and unrestricted use of antibiotics in animal production has led to a surge in antibiotic resistant bacterial strains. The poultry industry is steadily headed for a post antibiotic era, thus fuelling the search for alternative treatments for bacterial infections. One of these alternative treatments is the use of quaternary ammonium compound (QAC) based disinfectants. QACs are cationic surface active detergents widely used in the poultry industry because of their low relative toxicity and good antibacterial properties. Reports on QAC resistant bacteria have been on the increase in the food industry and thus studies on bacterial resistance to QACs are on the increase.

In order to try and understand disinfectant resistance, it is important to gain a better understanding of the mode of action of QAC based disinfectants of bacterial cells, particularly in the light of a pending post antibiotic era. In order to do this, bacteria treated with DDAC were examined using Scanning electron microscopy (SEM) and Nano Scanning Auger Microscopy (NanoSAM). *Staphylococcus aureus* strain ATCC 2357 treated with DDAC revealed protuberances or “bleb” formations on their cell walls when observed with SEM. The DDAC treated cells were further investigated using NanoSAM. NanoSAM is the combination of Scanning Auger Microscopy (SAM) and etching with an Argon (Ar^+) gun. SAM has the ability to perform semi-quantitative elemental analysis on extremely small volumes while visualizing the sample with SEM. Using NanoSAM technology we were able to visualize morphological changes caused by the disinfectant that SEM could not show. Clear evidence of a disruption of the cell membrane and the leaking out of cellular content was obtained.

Resistance to QAC has been attributed to the presence of the *qac* resistance genes, *smr, qacJ, qacG, qacH*. During this study the presence of the *qac* resistance genes could be correlated to the degree of resistance QACs. The *qac* resistance genes were identified using conventional PCR in strains that displayed higher tolerance to the different QACs. No *qac* resistance genes where identified in the susceptible strain ATCC 25923 using conventional PCR even though this strain was resistant to one of the QACs, benzalkonium chloride. An increased resistance to the different QACs could not be attributed to the presence of one specific *qac* resistance gene.

Real time PCR was introduced in this study since it is a technique known to be more sensitive than conventional PCR. Using real time PCR, it was revealed that all the bacterial strains contained more than one *qac* resistance gene. Interesting results were obtained with the susceptible strain ATCC 25923, where *qac* resistance genes were detected with real time PCR, while these genes were not detected using conventional PCR. Similar results were obtained with the Avian pathogenic *Escherichia coli* (APEC) strain isolated from poultry pens. After detecting the presence of the genes, the focus of the study changed to investigate the levels of expression of one of the *qac* resistance genes, *smr*. The expression study was performed using relative quantitative real time PCR. The hypothesis was that expression is increased when QACs are present in a culture medium. During the study it was revealed that there was no significant difference in the expression of the *qac* genes during cultivation in the presence of different QACs. There was, however a difference in the expression of the different strains tested where the *smr* was only expressed in the strain VB4-smr and not in the strains VB3-qacJ and ATCC 25923 during cultivation in the QAC didecyldimethylammonium chloride (DDAC). An additional hypothesis was subsequently formed. This hypothesis postulates that there is a difference in the expression of the *smr* gene over a time interval. During this study it was revealed that there was a significant difference in the expression of *smr* cultivated in different concentrations of DDAC, but there was no significant difference in the expression over a time interval.

From this study, it has been established that *qac* resistance genes are present in various bacteria and that using the more sensitive real time PCR test, additional *qac* genes were found in most of the strains. From the expression studies, it can be concluded that the levels of resistance is not merely related to the presence or absence of a particular *qac* resistance gene. It was also established that resistance is also not always directly related to increased levels of expression of a particular *qac* resistance genes. From this study, it is evident that resistance to disinfectants is multi factorial and substantial additional research is required to fully understand resistance to disinfectants.

Key words: DDAC, NanoSAM, PCR, Real time PCR, Relative quantitative PCR, resistance, protuberances, *Staphylococcus aureus*.

Opsomming

Die wydverspreide en onbeperkte gebruik van antibiotika in diereproduksie het tot 'n toename in antibiotiese weerstand teen bakteriese stamme gelei. Die pluimveebedryf is stadig besig om na 'n post antibiotiese era te beweeg, dus dryf dit die soektog na alternatiewe behandelings vir bakteriese infeksies. Een van hierdie alternatiewe behandelings is die gebruik van kwaternêre ammonium verbinding (KAV) gebaseerde ontsmettingsmiddels. KAV is kationiese oppervlak aktiewe skoonmaakmiddels wat wyd gebruik word in die pluimveebedryf as gevolg van hulle lae relatiewe toksisiteit en goeie antibakteriese eienskappe. Toename in KAV weerstandbiedende bakterieë gevalle word gerapporteer in die voedselbedryf, dus neem die studies op bakteriële weerstand teen KAVs toe.

Ten einde om weerstandbiedendheid teen ontsmettingsmiddel beter te verstaan, is dit belangrik om 'n beter begrip van die werking van KAV gebaseerde ontsmettingsmiddels teenoor bakteriële selle te verkry. Ten einde dit te kan doen, is bakterieë wat behandel is met DDAC ondersoek met behulp van Skandering elektron mikroskopie (SEM) en Nano Scanning Auger Mikroskopie (NanoSAM). *Staphylococcus aureus* stam ATCC 2357, wat behandel is met DDAC, openbaar uitsteeksels of "blaas" formasies op hulle selwande wat deur SEM waargeneem is. Die DDAC behandelde selle was ondersoek met behulp van NanoSAM. NanoSAM is die kombinasie van die Scanning Auger Mikroskopie (SAM) en geskil met behulp van 'n argon geweer. SAM het die vermoë om die semi-kwantitatiewe elementele analise uit te voer op baie klein volumes, terwyl die monster met SEM gevisualiseer word. Morfologiese veranderinge wat deur die ontsmettingsmiddel veroorsaak was, kon met die NanoSAM tegnologie gevisualiseer word, waar dit nie met SEM moontlik was nie. Daar was duidelike bewyse dat die selmembraan ontwrig was wat tot die lekking van sellulêre inhoud gelei het.

Weerstandbiedendheid teen KAV word toegeskryf aan die teenwoordigheid van die *kav* wee stands gene, *smr*, *qacJ*, *qacG*, *qacH*. Tydens hierdie studie was die verband tussen die teenwoordigheid van die *kav* weerstand gene en die minimum inhiberende konsentrasies (MIK) teen verskeie KAVs getoets. Die *kav* weerstand gene was geïdentifiseer met behulp van konvensionele PKR in stamme wat hoë toleransie vertoon het teen die verskillende KAVs. Geen *kav* weerstand geen was geidentifiseer

in die vatbare stam ATCC 25923 met behulp van konvensionele PCR, alhoewel hierdie stam bestand is teen een van die KAVs, benzalkoniumchloride. Verhoogde weerstand teen die verskillende KAVs kan nie toegeskryf word aan die teenwoordigheid van ‘n spesifieke *kav* weerstand geen nie.

Real-time PKR is in hierdie studie gebruik, aangesien die 'n tegniek meer sensitief as konvensionele PKR is. Met real-time PKR, was dit aan die lig gebring dat al die stamme van bakterieë bevat meer as een *kav* weerstand gene. Interessante resultate was verkry met die vatbare stam ATCC 25923, waar *kav* weerstand gene met real time PKR opgespoor was, terwyl hierdie gene nie opgespoor was met konvensionele PKR nie. Soortgelyke resultate is verkry met die voël patogeniese *Escherichia coli* (APEC) stam geïsoleerd uit pluimvee penne. Na die opsporing van die gene, het die fokus van die studie verander, na die bestudering van die uitdrukkings vlakke van een van die *kav* weerstand gene, *smr*. Die uitdrukking studie is uitgevoer deur gebruik te maak van relatiewe kwantitatiewe real time PCR. Die hipotese was dat die uitdrukking verhoog word wanneer KAVs teenwoordig is in die kultuur medium. Die studie het dit aan die lig gebring dat daar geen beduidende verskil in uitdrukking van die kav geen was tydens die kweking in die teenwoordigheid van verskillende KAVs. Daar was egter 'n verskil in die uitdrukking van die verskillende stamme wat getoets was, waar die smr geen slegs uitgedruk was in die stam VB4-smr en nie in die stamme VB3-qacJ en ATCC 25923 tydens kweking in die KAV didecyldimethylammonium chloried (DDAC). 'n Bykomende hipotese is daarna gevorm. Hierdie hipotese postuleer dat daar 'n verskil is in die uitdrukking van die smr gene oor' n tydinterval. Tydens hierdie studie was dit aan die lig gebring dat daar 'n beduidende verskil in die uitdrukking van SMR is wat gekweek was in verskillende konsentrasies DDAC, maar daar was geen betekenisvolle verskil in die uitdrukking oor' n tydinterval.

Uit hierdie studie, is vasgestel dat die qac weerstand gene teenwoordig is in verskeie bakterieë en dat die gebruik van die meer sensitiewe real time PCR-toets, addisionele kav gene was in die meeste van die stamme gevind. Van die uitdrukking studies, kan dit afgelei word, dat die vlakke van weerstand nie net verwant is aan die teenwoordigheid of afwesigheid van 'n bepaalde kav weerstand geen. Daar is ook vasgestel dat die weerstand nie altyd direk verband hou met verhoogde vlakke van uitdrukking van 'n bepaalde kav weerstand geen nie. Uit hierdie studie, is dit duidelik dat die weerstand teen ontsmettingsmiddels multi-faktoriaal is en aansienlike bykomende navorsing is nodig om ten volle weerstand te ontsmettingsmiddels te verstaan.

Sleutel woorde: DDAC, NanoSAM, PKR, Real-time PKR, Relatiewe kwantitatiewe PKR, weerstand, uitsteeksels, *Staphylococcus aureus*.

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Appendix I

>qacG

CGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGA
AACCCCCTAACACTTAGCACTATCGTTTACGGCGTGGACTACCAGGGTATCTAATTCTG
GTTGAATTCCCACGCCTTCCCCCATCCACGTCCGGTACCAACCAAGAAGTTCCCTTCG
GCAATTGTGGTTCTTCCTAACTTTGCCCCATTCCACCGCTCCCCATGGAATTCCCCTTT
CTTCTTCGGCACTCAAGTTTTCCAGTTTCCAAGGACCCTCCACG

>qacH

CAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAG
GGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTAT
CTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTC
GCCTTCGCCACTGGTGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATTC
CACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCG
TGGGCTTTCACATCAGACTTAAAAAACCGCCTACGCGCGCTTT

>qacJ

TCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAA
GGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTA
TCTAATCCTGTTTGATCCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGT
CGCCTTCGCCACTGGTGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATT
CCACTTTCCTCTTCTGCACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCC
GTGGGCTTTCACATCAGACTTAAAAAA

>smr

ATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGT
ACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCT
AACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGATC
CCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGT
GTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATTCCACTTTCCTCTTCTGC
ACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGG

>16SrRNA resistant samples

CATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCG
TACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCC
TAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGAT
CCCCACGCTTTCGCACATCAGCGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGG
TGTTCCTCCATATCTCTGCGCATTTCACCGCTACACATGGAATTCCACTTTCCTCTTCTG
CACTCAAGTTTTCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCA
GACTTAAAAAACCGCCTACGCGCGCTTTACGCCC

Appendix II

Table II.1 C_T and T_M values for standard curves generated for Housekeeping genes, *rho, proC and gyrA* as well as *smr* gene performed in triplicate

Dilutions	*proC*		*gyrA*		*rho*		*smr*	
	C_T	T_M	C_T	T_M	C_T	T_M	C_T	T_M
10^0	20.54	80.34	19.25	80	18.61	81.38	10.64	78.72
10^{-1}	24.31	80.36	23.27	80.17	22.25	81.52	14.58	78.70
10^{-2}	27.86	80.40	26.69	80.24	25.57	81.40	17.88	78.60
10^{-3}	>30	81.14	> 30	81.12	> 30	81.57	21.57	78.53
10^{-4}	>30	81.52	> 30	81.86	> 30	82.20	25.61	78.64
10^{-5}		69.60	> 30	82.09	> 30	82.76		
10^0	20.44	80.27	19.85	80.13	19.16	81.37	9.75	78.49
10^{-1}	24.13	80.21	23.03	80.02	22.28	81.33	15.22	78.48
10^{-2}	28.25	80.34	26.91	80.28	25.60	81.36	19.09	78.47
10^{-3}	>30	80.90	> 30	81.01	> 30	81.52	21.79	78.48
10^{-4}	>30	81.45	> 30	81.33	> 30	82.22	25.71	78.54
10^{-5}			> 30	82.11	> 30	82.43		
10^0	21.34	80.25	19.31	80.13	18.63	81.35	9.76	78.50
10^{-1}	24.46	80.17	23.20	80.25	22.68	81.04	14.74	78.48
10^{-2}	28.52	80.34	26.61	80.96	25.56	81.50	17.97	78.55
10^{-3}	>30	81.14	> 30	81.80	> 30	81.32	22.74	78.68
10^{-4}	>30	81.67	> 30	74.10	> 30	82.39	25.84	78.58
10^{-5}		81.70						

Table II.2 C_T and T_M values for genes during relative quantitative real time PCR. *smr* gene expression study during the cultivation of strains VB4_smr, VB3_qacJ and ATCC in different concentrations of DDAC.

DDAC treatment	*S. aureus* strains	Genes	First Run		Second Run	
			C_T	T_M	C_T	T_M
No QAC	VB4_smr	*gyr A*	17.66	80.65	16.77	80.78
		proC	17.49	80.63	19.33	80.79
		rho	17.44	81.89	17.22	82.07
		smr	12.47	78.98	11.30	79.05
	VB3_qacJ	*gyr A*	15.65	80.57	14.96	80.73
		proC	17.63	80.33	16.96	80.52
		rho	15.71	81.87	15.52	82.02
		smr	26.73	78.57	27.29	78.25
	ATCC 25923	*gyr A*	17.11	80.32	16.32	80.34
		proC	22.27	81.32	22.46	81.03
		rho	17.65	81.84	17.23	81.92
		smr	25.62	78.36	30	77.47
	Calibrator	*gyr A*	17.33	79.82		
		proC	22.27	79.97		
		rho	17.85	81.10		
		smr	9.85	78.43		
0.2	VB4_smr	*gyr A*	14.90	80.11	19.25	80.67
		proC	16.96	80.43	18.85	80.50
		rho	14.93	81.77	19.80	81.92
		smr	8.76	78.94	9.32	79.04
	VB3_qacJ	*gyr A*	14.76	80.60	14.84	80.75
		proC	16.76	80.61	16.76	80.15
		rho	14.52	81.94	15.15	82.03
		smr	25.97	78.71	26.07	78.75
	ATCC 25923	*gyr A*	14.71	80.25	14.78	80.32
		proC	19.87	81.29	20.91	81.03
		rho	15.01	81.78	15.66	81.96
		smr	26.24	78.81	26.67	78.66
1	VB4_smr	*gyr A*	16.08	80.53	15.25	80.59
		proC	18.16	80.53	17.63	80.65
		rho	15.92	81.84	15.87	81.89
		smr	9.51	78.96	8.63	78.93
	VB3_qacJ	*gyr A*	14.97	80.50	14.92	80.65
		proC	17.11	80.54	16.92	80.64
		rho	15.18	81.81	16.92	81.97
		smr	26.92	78.78	26.64	78.84

Table II.3 C_T and T_M values for genes during relative quantitative real time PCR. smr gene expression study during the cultivation of strains VB4_smr in different concentrations of DDAC for different time intervals in duplicate.

Time (h) intervals	Genes	DDAC Treatments			
		0		0.2	
		C_T	T_M	C_T	T_M
2	gyr A	13.97	80.58	16.58	80.46
	proC	16.72	80.68	20.97	80.57
	rho	13.81	81.85	15.66	81.79
	smr	7.68	79.11	14.51	78.81
4	gyr A	13.41	80.67	16.31	80.29
	proC	15.61	80.65	20.72	80.62
	rho	13.62	81.93	16.46	81.73
	smr	6.52	79.14	16.64	78.76
8	gyr A	13.17	80.63	17.94	80.57
	proC	15.26	80.65	22.49	80.45
	rho	13.54	81.82	18.33	81.69
	smr	5.85	79.00	14.60	79.01
22	gyr A	13.63	80.66	20,14	80.54
	proC	15.88	80.61	23.85	80.46
	rho	14.02	81.79	19.52	81.78
	smr	6.38	79.02	17.45	78.92
2	gyr A	13.46	80.65	16.31	80.77
	proC	16.31	80.58	20.98	80.76
	rho	13.99	81.98	15.62	81.83
	smr	7.16	79.03	14.15	79.02
4	gyr A	12.80	80.76	18.04	80.72
	proC	14.77	80.89	21.54	78.93
	rho	13.07	81.92	16.44	81.96
	smr	6.03	79.13	14.95	79.12
8	gyr A	12.45	79.19	18.92	80.48
	proC	14.44	80.89	23.07	80.49
	rho	13.14	81.92	18.56	81.76
	smr	5.69	79.19	15.79	78.97
22	gyr A	13.07	80.70	21.11	80.51
	proC	15.13	80.81	24.89	80.54
	rho	13.78	81.91	20.45	81.75
	smr	5.73	79.14	18.71	79.

ANOVA: Single factor analysis

Summary

Groups	***Count***	***Sum***	***Average***	***Variance***
No QAC	3	0.50923	0.169743	0.086384
0.2 µg ml DDAC	3	1.355376	0.451792	0.612279
1 µg ml DDAC	3	0.83519	0.278397	0.232501

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	0.121423	2	0.060711	0.195599	0.827384	5.143253
Within Groups	1.862328	6	0.310388			
Total	1.983751	8				

ANOVA Two-Factor with replication

SUMMARY	2h	4h	8h	22h	Total
no QAC					
Count	2	2	2	2	8
Sum	11.95002	23.10369	29.26255	27.04049	91.35675
Average	5.975008	11.55184	14.63127	13.52025	11.41959
Variance	28.27248	103.9103	135.2076	155.183	73.05008
0.2 DDAC					
Count	2	2	2	2	8
Sum	0.224285	0.068453	0.078767	0.013688	0.385192
Average	0.112142	0.034226	0.039383	0.006844	0.048149
Variance	0.006298	0.000752	0.000369	8.89E-06	0.002796
Total					
Count	4	4	4	4	
Sum	12.1743	23.17214	29.34132	27.05418	
Average	3.043575	5.793035	7.335329	6.763546	
Variance	20.88399	78.8555	116.0437	112.5983	

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Sample	517.239	1	517.239	9.792004	0.014032	5.317655
Columns	43.46436	3	14.48812	0.274279	0.842429	4.066181
Interaction	45.32508	3	15.10836	0.286021	0.834319	4.066181
Within	422.5807	8	52.82259			
Total	1028.609	15				