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Full Length Research Paper

The influence of Didecyldimethylammonium Chloride on the morphology and elemental composition of *Staphylococcus aureus* as determined by NanoSAM

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Didecyldimethylammonium chloride (DDAC) is a Quaternary Ammonium Compound (QAC) disinfectant often used in the poultry industry to disinfect hard surfaces. DDAC is a membrane active agent and causes the leakage of important intracellular material. Understanding the mode of action and possible resistance is important; in particular, the pending post antibiotic era that the poultry industry is facing. *Staphylococcus aureus* strain ATCC2357 treaded with DDAC revealed protruberances or bleb formations on their cell walls when observed with scanning electron microscopy. The DDAC treated cells were further investigated using NanoSAM. This technology showed morphological changes as well as structural detail on control cells caused by the disinfectant that scanning electron microscopy could not. NanoSAM also showed a decrease in the elemental intensities during the etching of the cells treated with QAC. This proved that QAC leads to the leakage of cellular material.

Key words: Quaternary ammonium compound, *Staphylococcus aureus*, nano scanning electron microscopy.

INTRODUCTION

Antibiotics have been used at sub-therapeutic levels as growth promoters in animals as early as their discovery (Feighner and Dashkevicz, 1987). This widespread and unrestricted use of antibiotics led to the surge in disease cases related to antibiotic resistant bacterial infection (Threlfall, 2002). Subsequently, the use of antibiotics as growth promoters was banned soon after the increase in prophylactic usage of therapeutic antibiotics against bacterial infections in animals (Casewell et al., 2003). More restrictions on the use of antibiotics have been imposed and this placed emphasis on the search for possible alternatives for use in animal health (Joerger, 2003; Nelson et al., 2007; FDA, 2005). Alternative treatment methods include bacteriocins, small antimicrobial peptides and bacteriophages (Joerger, 2003). These methods seem promising, but they are still in the developmental stages and the safe application of these methods is uncertain.

Another option for the control of bacterial diseases in poultry in a post-antibiotic era is the continual disinfection program, where a non-toxic disinfectant has been used continually in poultry production where a reduction in bacterial loads has been recorded (Bragg and Plumstead, 2003). This non-toxic disinfectant (Virukill®) is based on a modified Quaternary ammonium compound (QAC).

QAC based disinfectants play an important role in veterinary medicine and in the control of animal diseases (Bjorland et al., 2005). QACs are cationic surface active

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detergents widely used for the control of microorganisms in clinical and industrial environments. The lack of selective toxicity and target specificity makes them different from antibiotics (Denyer and Stewart, 1998). QACs adsorb to the relatively anionic bacterial cell walls where they diffuse through the cell wall and bind to the cytoplasmic membrane (Hegstad et al., 2010; Iannou et al., 2007). Here they are thought to cause the disorganisation of the membrane which results in the leakage of the intracellular material ultimately causing cell death (Iannou et al., 2007).

The basic use of QAC based agents is for the disinfection of surfaces and is generally used at concentrations noticeably higher than the minimal inhibitory concentrations (MICs) (White and McDermott, 2001). The possibility still remains that some bacteria are exposed to low concentrations that allow for their survival, resulting in the development of resistance against these antibacterial agents (Chapman, 2003; Langsrud et al., 2003; Russel, 2002; White and McDermott, 2001). The widespread use and, to a degree, the misuse of antimicrobial agents ensure selective pressure on bacteria that are innately capable of acquiring genes enabling them to survive harsh environments (Sidhu et al., 2002; White and McDermott, 2001).

In order for an antibacterial agent to be effective, it needs to gain entry into the cell. Intrinsic bacterial resistance is largely a function of the chemical composition and structure of the cell surface while the acquired resistance usually occurs by means of mutations, through the acquisition of foreign resistance genes (Chapman, 2003; Langsrud et al., 2003; Russel, 2002; White and McDermott, 2001). Bacteria usually acquire these foreign resistance genes via plasmids and transposons that contain them (Chapman, 2003; Langsrud et al., 2003; Russel, 2002; White and McDermott, 2001). Transposons are mobile DNA elements and have been shown to possess a number of genes encoding resistance to QACs and antibiotics (White and McDermott, 2001). These elements are often spread by incorporation into plasmids and have contributed to the rapid spread of antibiotic resistance (Gillings et al., 2008a, b; 2009a). QACs and antibiotics are, in certain cases, used in the same area. In most cases QACs would be used to disinfect the poultry pens while antibiotics are used to control disease outbreak. Concerns have been raised regarding disinfectant resistance, which may contribute to antibiotic resistance by co-selection of antibiotic resistance genes that reside on the same transposon as the disinfectant resistance genes (Gaze et al., 2005; Gillings et al., 2009b; Sidhu et al., 2001). Thus, the presence of sub-MIC concentrations of QAC may not only induce bacterial resistance to QAC, but could also result in increased antibiotic resistance (Sidhu et al., 2001, 2002). It is imperative 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 worldwide poultry industry is facing.

Bacterial cells, like other organisms, have survival mechanisms which emerge when affected by environmental stress factors (Choi et al., 2003). It has been observed that some unicellular organism can die in a way that resembles apoptosis, or in this case cell necrosis, where biochemical events lead to unusual cell changes when affected by compounds such as QACs (Benndorf et al., 2004). These changes include blebbing, swelling, bursting and spilling of intracellular content, but the molecular mechanisms of bacterial apoptosis and necrosis are not clearly understood (Raff, 1998). One method of apoptosis that has been documented is phage exclusion (Georgiou et al., 1998; Gottesman, 1998). This is when bacterial apoptosis shares similarities to the caspase-dependant apoptosis in animal cells, but with unrelated proteases being involved in the two processes (Raff, 1998). Bacterial apoptosis may be an important factor during antibacterial activity and could also play a role in the process that allows bacteria to become resistant to these substances. Attempts have been made to record the morphological changes in bacteria after treatment with QACs (Kourai et al., 1994; Yoshimatsu and Hiyama, 2007). Membrane active compounds can induce damage on the cell membrane and its permeability (Al-Adham et al., 1998). Particle-like debris from bacterial membranes was observed after treatment with QACs (Wattanaphansak et al., 2010). Kourai observed what they termed “bleb” formations on *Escherichia coli* cells and later Yoshimatsu made the same observation on *Staphylococcus aureus* cells treated with high concentrations of QAC (Kourai et al., 1994; Yoshimatsu and Hiyama, 2007). Thus, disinfectants might have the potential to lyse bacterial cells (Wattanaphansak et al., 2010).

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). SAM is a powerful tool used in the semi-quantitative elemental analysis on extremely small samples and is usually used for the near-surface analysis of conductor and semi-conductors while the sample is visualised by Scanning Electron Microscopy (SEM) (Calvo-Barrio et al., 2001; Hochella et al., 1986). 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-Barrio et al., 2001). 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 ultrastucture of the surface of the cells respectively. 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 while 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 that QACs have on *S. auerus* cells.

MATERIALS AND METHODS

Bacterial strains

S. aureus strain ATCC 25923 was (obtained from the University of the Free State, South Africa bacterial culture collection) cultivated in tryptic soy broth (Merck, Darmstadt, Germany) at 37°C for 16 h.

Minimal inhibitory concentrations

The minimal inhibitory concentration (MIC) of *S. aureus* strain ATCC 25923 to Didecyldimethylammonium Chloride (DDAC) (Uniquat 2280, Lonza, USA) was determined using a micro titer assay. Serial dilutions of DDAC were made in Mueller-Hinton broth (Merck, Darmstadt, Germany) and 2×10^8 cfu ml^{-1} cells were inoculated and incubated at 37°C for 16 to 18 h. The MIC was the lowest concentration at which no growth was observed.

Quaternary ammonium compound treatment

Cells were harvested by centrifugation at 7000 *g* for 5 min at 4°C. Cells were washed twice in 3-Morpholinopropanesulfonic acid (MOPS) buffer (Fluka analytical, Steinheim, Germany) and resuspended in MOPS buffer supplemented with 0.4 or 20 g/l DDAC incubated for 10 min and prepared for SEM. Cells used as a control were resuspended in MOPS buffer without DDAC and prepared for SEM in the same manner as described below.

Scanning electron microscopy

Cells were fixed for SEM by adding 6% (v/v; 0.1 ml/L) gluteraldehyde in sodium phosphate buffer and incubated for 2 h. After incubation, cells were washed with the same buffer to remove excess aldehyde fixative. Post-fixation was performed with 4% (m/v) buffered osmium tetroxide (Merck, Darmstadt, Germany) and incubated for 1 h after which cells were washed to remove excess osmium tetroxide. Cells were harvested on 0.2 µm filter and dehydrated using an ethanol sequence of 50, 70, and 95% for 10 min per step and followed by two 30 min steps of 100%. Drying was performed by using a critical point dryer. Thereafter specimens were mounted on stubs, coated with gold and viewed using the SEM (Shimadzu SSX-550 Superscan, Tokyo, Japan).

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 analyses was set at: 2.34A 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 analyses and SEM imaging with an electron beam diameter of 27 nm. The upper pressure of the electron gun unit was 8.8 E-10 Torr and the pressure in the main chamber was 2.29 E-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 analyses were obtained by using 10 cycles per survey, 1 eV/step and 20 ms per step. The nanoprobe was also equipped with an Ar^+ ion sputter gun set at: 2 kV beam voltage, 2 µA ion beam current and a 1 × 1 mm raster area, giving a sputter rate of 27 nm/min for the first 3 min, and then reduced to 8.5 nm/min. 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.

RESULTS

The MIC for *S. aureus* against DDAC was determined to be 0.59 mg/l. Subsequently the cells were treated with DDAC at a sub-MIC concentration of 0.4 mg/l in order to observe possible morphological changes induced by the QAC. The control cells as well as the cells treated with DDAC appeared typically spherical (Figure 1). The cells treated with sub-MIC levels of QAC did not show any significant difference when compared to the control cells and subsequently the cells were treated with 20 g/l DDAC. The DDAC treated cells displayed an increase in nodules on the cell surface and an apparent leakage of cell material was observed (Figure 1b). Cells in process of cell division were abundant in the control sample (Figure 1a). This was not observed with the DDAC treated cells.

The ion gun was used successfully to make targeted sputtered etching depth profiles of up to 310 nm into the cells (Figure 2a-i). Etching proceeds through the control cells and revealed a smooth layer underneath the gold (Figure 2b). The sub-MIC level of DDAC did not cause any significant changes in the cell, thus the experiment was repeated exposing cells to 20 g/l DDAC. Etching through the DDAC treated cells revealed a smooth layer and a distinct hole where the nodule was under the gold layer (Figure 2e).

SAM elemental colour maps indicated a high gold (Au) intensity (Figure 2g, Au indicated as green, corresponding to Figure 2d) as the sample was coated in Au during preparation which permits the sample charge conductivity. As etching proceeds, carbon (C) and oxygen (O) were revealed (C indicated in blue and O indicated in red; Figure 2h corresponding to Figure 2e). Carbon intensity gradually increases as etching continues, but some Au still remains as the ion gun could not reach these sections of the cells (Figure 2i corresponding to Figure 2f). No trace of Chlorine (Cl an indicator of QAC) was observed in the QAC treated cells, both in the 0.4 and 20 g/l DDAC treated cells. As the field of view (FOV) was increased and a larger surface on the stub scanned, cells that appeared empty and shrunken were observed in the QAC treated cells (Figure 3b) which were not observed in the control cells (Figure 3a).

During the sputtering experiment an auger point analysis spectrum was taken at specific sites on the bacterial cells to obtain quantitative assessment of the elemental composition as etching proceed (Figure 4). A sharp decrease in the intensity of Au was evident in the control cells followed by a sharp increase in carbon (Figure 4b, Target 3). There was a gradual decrease in

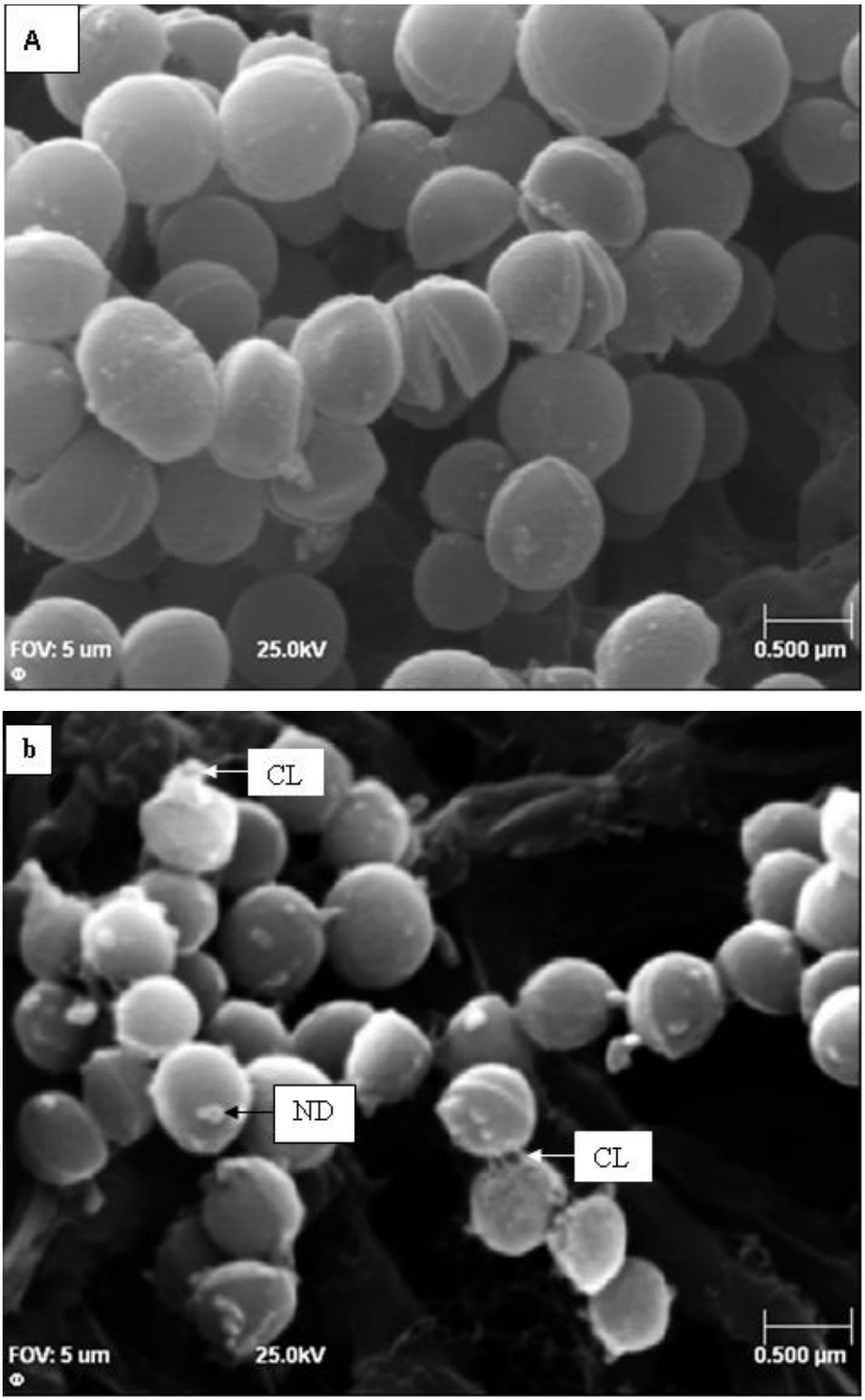
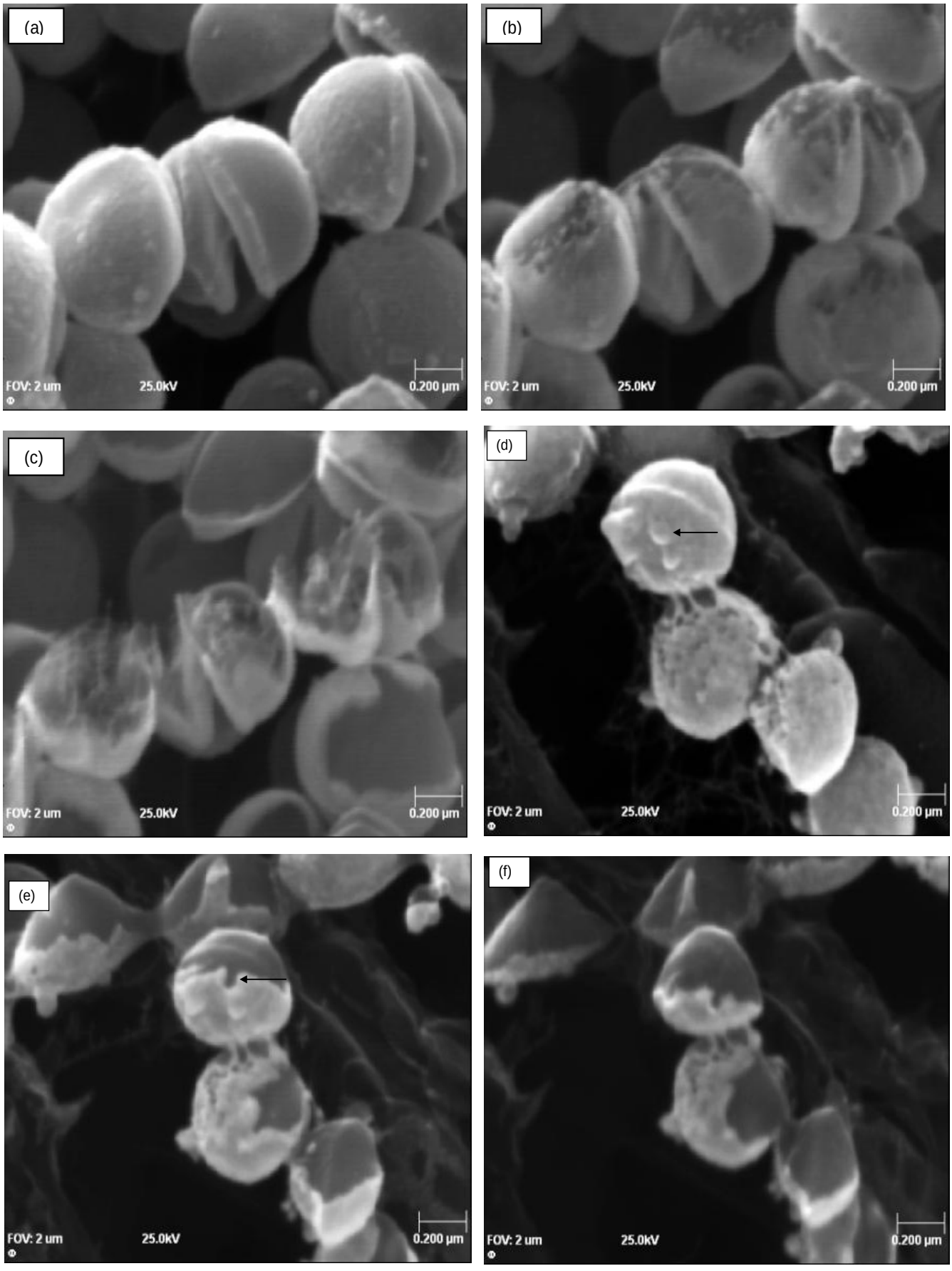


Figure 1. Scanning electron micrographs of *Staphylococcus aureus* strain ATCC 25923 indicating the effect didecyldimethylammonium chloride (DDAC) had on the cells morphology (a) control cells containing no DDAC and displaying the normal spherical shapes of *S. aureus* (b) Cells treated with 20 g/l DDAC for 10 min. The arrows indicated where cell leakage (CL) and nodule formation (ND) was observed when the *S. aureus* cells were incubated with DDAC.


(a)
FOV: 2 um
25.0kV
0.200 µm
(b)
FOV: 2 um
25.0kV
0.200 µm
(c)
FOV: 2 um
25.0kV
0.200 µm
(d)
FOV: 2 um
25.0kV
0.200 µm
(e)
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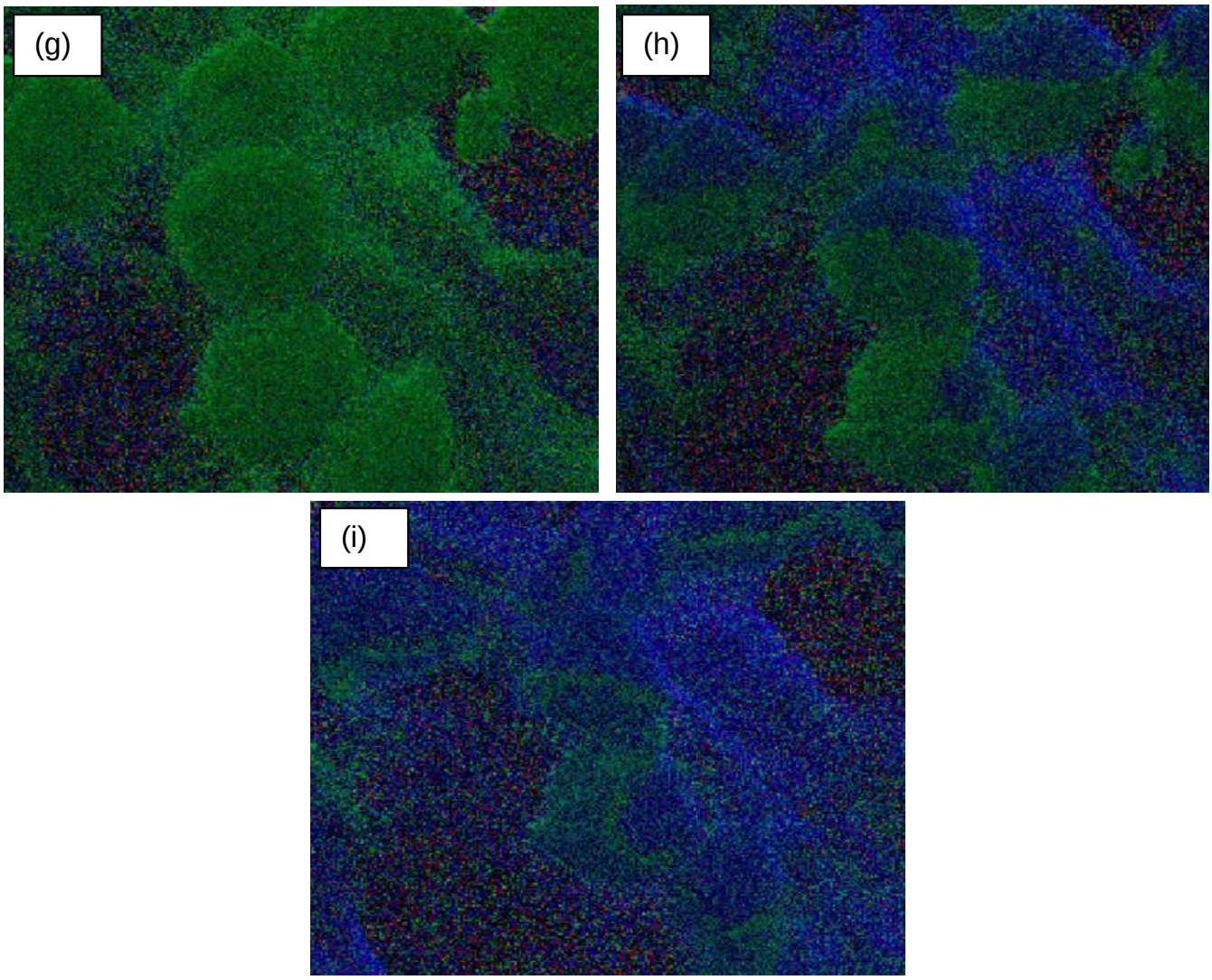

Figure 2. Scanning electron micrographs at different stages of sequential etching into *S. aureus* strain ATCC 25923 control cells without the presence of didecyldimethylammonium chloride (DDAC) (a – c), cells treated with 20 g/l DDAC for 10 min (d - f), colour maps of the various elements present in the sample (g - i). These colour maps corresponded to the etching micrographs (d - f) green – gold, blue – carbon and red – oxygen. The arrow indicates the nodule on the DDAC treated cell and the sequential etching through this nodule revealing a hole in the cell as indicated by the darker area partially surrounded with lighter area which was the gold still present on the cell wall.

the gold concentration of the DDAC treated cells with a corresponding gradual increase in the carbon at the specific sites (Figure 4d, Target 3). 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 profile was similar for all the other sites for both the control as well as the DDAC treated cells, indicating that the nodules are part of the cell wall and not foreign material. Only trace amount of chlorine (an indicator of DDAC as its chemical structure contains chlorine) was detected but it was too little to distinguish it from the background noise to be conclusive (Figure 4d). Trace amounts of nitrogen, oxygen and osmium was detected during sputtering.

DISCUSSION

The poultry industry is steering towards a post-antibiotic era with currently no means to control the growth of pathogenic bacteria. With all the problems of antibiotic resistance, attention is shifting towards the use of disinfectants as one of the potential controls of bacterial infections in poultry production. Therefore, it is important to fully understand the mode of action of these disinfectants and how bacteria are developing resistance

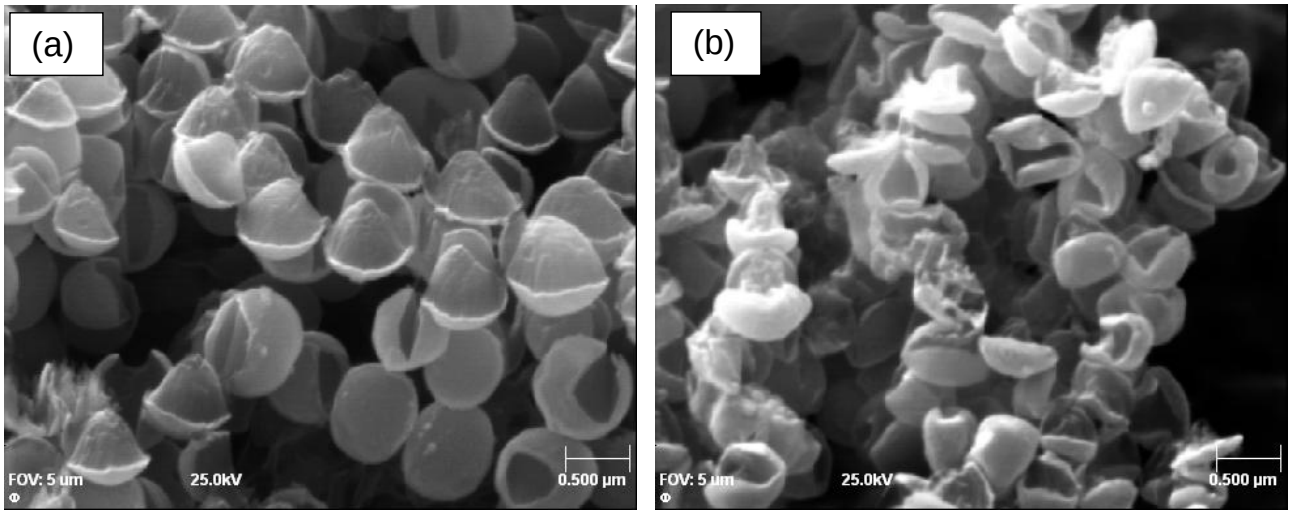

Figure 3. SEM micrographs at the end of etching trough *Staphylococcus aureus* strain ATCC 25923 cells in the (a) absence of didecyldimethylammonium chloride (DDAC) and (b) the presence of 20 g/l DDAC. The DDAC treated cells completely collapsed under the 25 kV of the argon gun where the control cells was still intact.

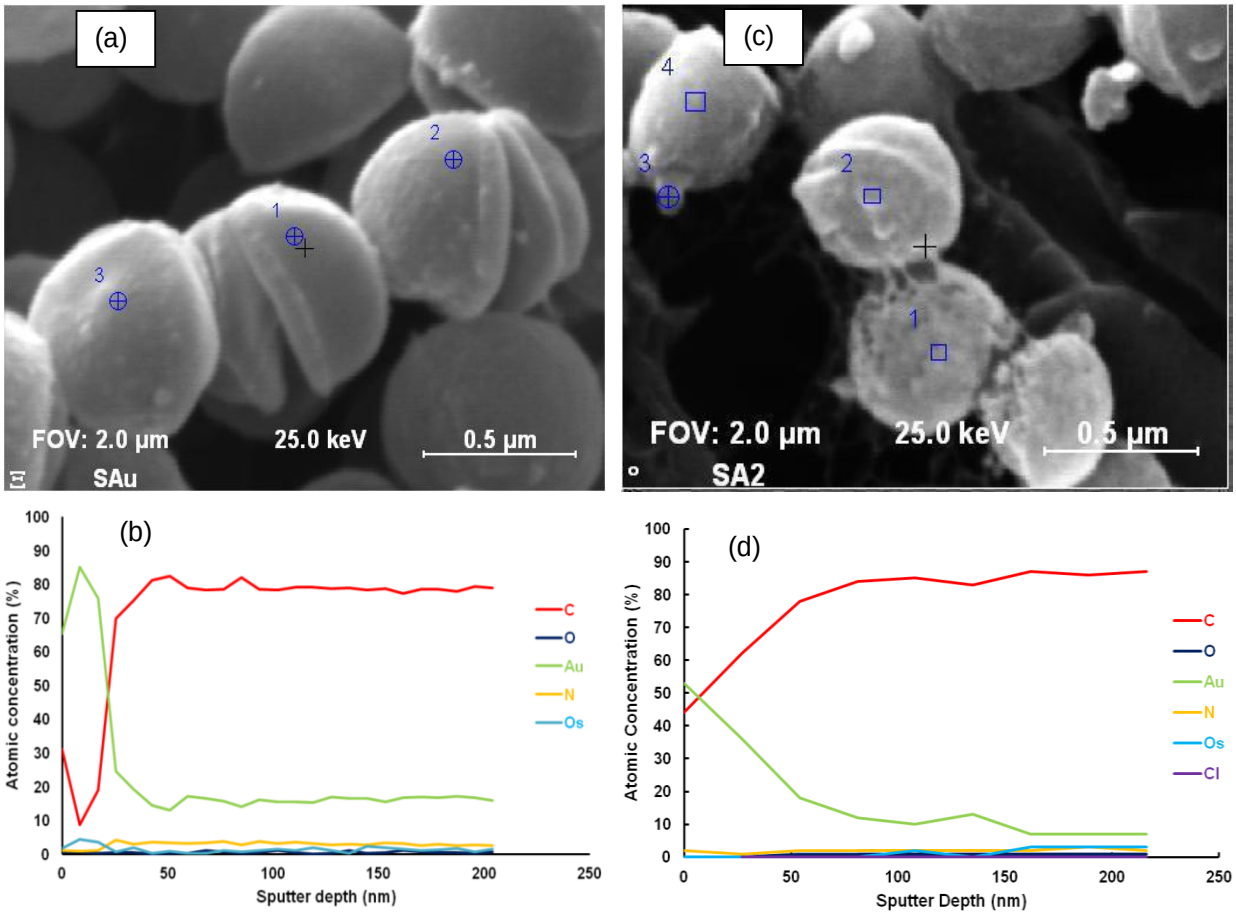

Figure 4. Elemental analyses through cells during sequential (a), SEM micrograph of control cells not treated with DDAC. The targets for elemental analysis are indicated by crossed circles. (b), A graph showing atomic concentration over sputter depth of control sample in a (Target 3). (c), SEM micrograph of DDAC treated cells. The targets for elemental analysis are indicated by crossed circles (d) A graph showing atomic concentration over sputter depth of cells treated with DDAC in c (Target 3).

against them.

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 and it was for the first time successfully applied on *S. aureus* cells (Swart et al., 2010). 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. This enabled the detection of the 3-D nanostructure of cells and DDAC-treated cells. From the results (Figure 2a-i) we conclude that DDAC causes morphological changes in *S. aureus* cells. Treatment with DDAC caused the formation of nodules on the cell walls and ultimately caused leakage of the cellular material. Yoshiatsu and Hiyama (2007) made a similar observation with *E. coli* cells where “bleb” formation followed by the leakage of intracellular molecules was seen.

Elemental analysis was performed by focusing the nanoprobe on a specific target site on the sample which was bombarded with electrons resulting in the release of Auger electrons giving a specific energy profile (Calvo-Barrio et al., 2001). These profiles were specific for every element and due to all elements having a unique set of electron-binding energies, 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 the different elements in specific colours. A colour map of various elements was obtained for each sample surface analyzed (Figure 2g - l). Elemental composition depth profiles from all target sites revealed no significant differences between the various treated cells (results not shown). There was no chlorine detected in any of the samples which raised the question as to what happened to the QAC after the cells were exposed to it. One theory is that the QAC is pumped out of the cell and removed during preparation for SEM. Another theory is that the QAC is altered after penetration and the chlorine is expelled from the cells. Further research is necessary to understand what happens with the QAC after entering a cell.

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