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Proteomic analysis of the proteins released from *Staphylococcus aureus* following exposure to Ag(I)

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ABSTRACT

The silver ion (Ag(I)) has well established antimicrobial properties and is widely used in a variety of anti-bacterial ointments and plasters for the control of wound infections. Wounds are frequently colonised by the bacterium *Staphylococcus aureus* and the aim of the work presented here was to establish how *S. aureus* responded following exposure to Ag(I). Exposure of *S. aureus* to Ag(I) resulted in the release of a range of proteins from cells. Analysis of proteins released revealed a number of proteins associated with the stress response (e.g. alkaline shock protein, methionine sulfoxide reductase), virulence (e.g. signal transduction protein) and metabolism (e.g. lipase, acetate kinase, phosphoglycerate mutase). The release of toxins (e.g. α-hemolysin, bifunctional autolysin, leucocidin F) was decreased. These results indicated that, while silver is a potent antimicrobial agent, exposure of *S. aureus* to this metal results in the release of a variety of proteins from the cell. Many of the proteins showing increased release were antigenic and would have the potential to induce an inflammatory response at the site of infection and thus delay healing.

1. Introduction

The anti-microbial activity of silver has been known for many years and the Ag(I) ion is the active agent in many healthcare products (Lansdown, 2010). Central venous catheters impregnated with silver metal particles substantially reduced the incidence of catheter-associated infections (CAIs) in paediatric patients (Carbon et al., 1999). Such silver-impregnated catheters have been shown to prevent CAIs through a reduction in bacterial adherence by up to 40% (Bechert et al., 1999). There is a wide range of wound dressings incorporating silver available such as hydrogels, creams, foams, meshes, hydrocolloids and polymeric films (Thomas and McCubbin, 2003). Clinical investigations into the anti-bacterial nature of such silver-containing dressings have revealed the potent activity against *Pseudomonas aeruginosa,* vancomycin-resistant *Pseudomonas* species and methicillin-resistant *Staphylococcus aureus* (MRSA) (Ulker et al., 2005; Olsen et al., 2000). Silver has been widely used in the treatment of *S. aureus* infections and one of its primary modes of action has been suggested to involve disruption of bacterial membrane integrity (Randall et al., 2013). Other clinical benefits of silver wound dressings include a reduction in wound associated pain (Rustogi et al., 2005), increased epithelialisation of skin grafts (Demling and Desanti, 2002) and increased efficiency at preventing MRSA colonisation of burn wounds (Honari et al., 2011). The anti-bacterial nature of the Ag(I) ion has also been exploited by its incorporation into topical, anti-bacterial agents such as burn wound creams (e.g. silver sulphadiazine (SSD), silvadene) (Monafo and Freedman, 1987). Initial studies investigating the activity of silvadene on burn wounds colonised by *P. aeruginosa* revealed the superior activity of SSD against *P. aeruginosa*, with the cream also reducing post-burn destruction of the skin (Fox, 1968).

S. aureus is one of the main pathogens associated with infections of the skin and soft tissue (Kirby et al., 2002) and it can lead to more serious conditions such as toxic shock syndrome (TSS), scalded skin syndrome (SSS) and sepsis (Plano, 2004). Many factors contribute to the pathogenesis of *S. aureus* such as the presence of a capsule, the expression of adhesins, the secretion of various toxins and also immunomodulators. *S. aureus* produces a wide variety of cytotoxins which have potent effects on the cells of the host immune system (Dinges et al., 2000). *S. aureus* has been shown to be a very versatile pathogen and has been isolated from both hospital and community-acquired infections (García-Lara et al., 2005). *S. aureus* infection can be extremely difficult to control and up to 4% of cases may be fatal (García-Lara et al., 2005). The Ag(I) ion is highly active against a range of microorganisms through interactions with the cell wall which results in changes in conformation (Leaper, 2006). This allows the Ag(I) ion to penetrate the cells while causing cell leakage, denaturation and inactivation of proteins and essential enzymes, such as RNA- and DNA-ases, ultimately leading to cell death (Slawson et al., 1990).

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The action of the Ag(I) ion on the cell wall has been studied using the yeast *Candida albicans* and it has been shown to bind to cysteine residues of the enzyme, phosphomannose isomerase (PIM) and inhibit its activity (Lansdown, 2002). This enzyme plays an important role in cell wall biosynthesis (Wells et al., 1995) and inhibition can lead to leakage of important nutrients, such as phosphates and succinates, from the cytoplasm (Lansdown, 2006). The inhibitory action of the Ag(I) ion can be attributed to its strong binding affinity for thiol groups present in cell respiratory enzymes, its interaction with structural proteins and its binding with DNA bases which inhibits replication (Atiyeh et al., 2007).

The aim of the work presented here was to examine the effect of Ag(I) on the leakage of protein from *S. aureus* and to characterise the nature of the released proteins.

2. Materials and methods

2.1. Organism and culture conditions

A clinical *S. aureus* isolate from an urinary tract infection was employed. Cultures were grown on nutrient agar plates (Oxoid Ltd., Basingstoke, Hampshire, England) (28 g/L) at 37 °C for 24 h and kept at 4 °C for short-term storage. For liquid culturing, cells were cultured overnight in an aerated conical flask in an orbital shaker at 37 °C and 200 rpm in nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England) (13 g/L).

2.2. Assessment of protein release from S. aureus

Stationary phase *S. aureus* cells were harvested by centrifugation on a Beckmann GS-6 bench centrifuge at 1814*g* for 5 min. The cell pellets were washed twice in phosphate buffered saline (PBS, 5 ml) and resuspended in either dH_2O or $AgNO_3$ (3.0 μg/ml (17.6 μM), Sigma–Aldrich) and incubated at 37 °C and 200 rpm for a further 0.5, 1, 2 or 4 h. A concentration of 3.0 μg/ml was chosen as this had previously been established to be the MIC_{80} value (Smith et al., 2012). Cells were harvested as before and the supernatants collected. The quantity of protein released from the cells was assayed using the Bradford assay (Bio-Rad), with BSA (Sigma–Aldrich) as standard. The released protein was resolved by 1-D SDS–PAGE.

2.3. Isolation of released protein for 2D-SDS–PAGE analysis

Stationary phase *S. aureus* cells were exposed to the Ag(I) ions (3.0 μg/ml) for 2 h and harvested by centrifugation for 10 min at 1814*g* in a Beckmann GS-6 centrifuge. The cell pellet was washed twice with sterile phosphate buffer saline (PBS) and re-suspended in 2 mls of Lambert's breaks buffer (10 mM KCl (Sigma–Aldrich), 3 mM NaCl (Sigma–Aldrich), 4 mM $MgCl_2$ (Sigma–Aldrich), 10 mM 1, 4-piperazinediethanesulfonic acid (PIPES) (Sigma–Aldrich)). In addition, protease inhibitors, at a concentration of 10 μg/ml, were used (Leupeptin, Pepstatin A, Aprotinin and N-α-p-tosyl-L-lysine chloromethylketone hydrochloride (TLCK)). The cell suspensions were sonicated with two 10 s blasts using a soniprobe sonicator (Bandelin Sonopuls, HD 2200) to dislodge proteins loosely bound to the cell wall. This resulted in no loss in cell viability nor cell lysis (data not presented). The suspension was centrifuged at 239*g* for 4 min at 4 °C and the supernatant retained. Protein was precipitated by the addition of ice-cold acetone and storing the solutions at −20 °C overnight. The precipitated protein was collected by centrifugation on an Eppendorf 5417R centrifuge (17,949*g* for 30 min at 4 °C). The acetone was removed and the pellet allowed to air dry. Protein (300 μg) was separated by 2-D SDS–PAGE as previously described (Kelly and Kavanagh, 2010). All gels were performed in triplicate and the mean increase/decrease in abundance of protein was calculated.

3. LC/MS mass spectrometry analysis

Protein spots that exhibited altered intensities on 2-D gels of control and Ag(I)-treated cells were excised, washed and trypsin digested (Shevchenko et al., 2006). Samples were analysed on a 6340 Ion Trap LC/MS spectrometer (Agilent Technologies) using bovine serum albumin (BSA) as the external standard. The mass lists were generated using the search programme http://www.matrixscience.com and were blasted using the http://expasy.org/sprot/search programme. Mascot score values greater than 68 were considered significant ($p < 0.05$).

3.1. Statistical analysis

All experiments were performed on three separate occasions. Multiple comparisons of means were analysed using Fisher's least significant different test using PROC GLM of the SAS 9.1 statistical model. Differences were deemed significant with $p \leqslant 0.05$.

4. Results

4.1. Assessment of protein release from S. aureus cells as a result of Ag(I) ion exposure

Cells were exposed to Ag(I) ions as described and the extent of protein release was monitored by measuring the protein content of the supernatant (Fig. 1). There was an increase in protein release from cells exposed to Ag(I) ions which reached a peak at 2 h (96 ± 1.1 μg/ml in the control, 122 ± 1.1 μg/ml from Ag(I) ion treated cells ($p < 0.001$)). The released protein was also resolved by 1D SDS–PAGE and the resulting gel (Fig. 2) demonstrated that a wide range of protein is released from *S. aureus* when cells were exposed to 3.0 μg/ml Ag(I) for 0.5–4 h. The abundance of released proteins of molecular weight 25 kDa and 50 kDa, for example, reached a peak at 2 h exposure and thereafter declined.

4.2. Characterisation of changes in protein release following exposure to Ag(I) ions

S. aureus cells were exposed to Ag(I) ions (3.0 μg/ml) for 2 h as this had been determined to be the time of maximum protein

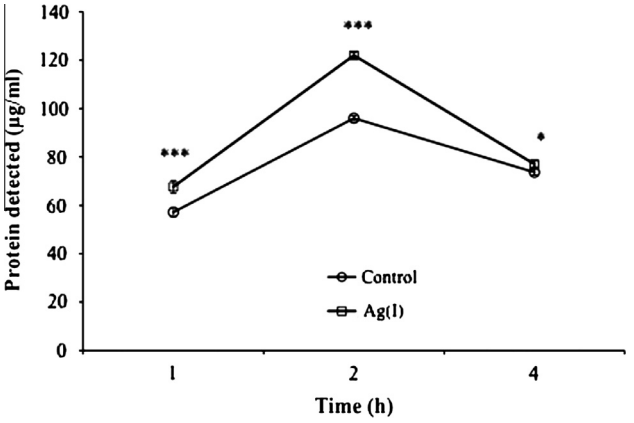

Fig. 1. The effect of Ag(I) ions on protein release from *S. aureus* cells. The release of protein from *S. aureus* cells, which had been exposed to either water or the MIC_{80} value of $AgNO_3$ (3.0 μg/ml), for 1, 2 or 4 h, was assessed as described. Differences in abundance were deemed statistically significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

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