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

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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 McCubin, 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 (Garcia-Lara et al., 2005). S. aureus infection can be extremely difficult to control and up to 4% of cases may be fatal (Garcia-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). 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 1814g for 5 min. The cell pellets were washed twice in phosphate buffered saline (PBS, 5 ml) and resuspended in either dH2O or AgNO3 (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 MIC80 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 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).

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 1814g in a Beckmann GS-6 centrifuge. The cell pellet was washed twice with sterile phosphate buffer saline (PBS) and resuspended in 2 ml of Lambert’s breaks buffer (10 mM KCl (Sigma–Aldrich), 3 mM NaCl (Sigma–Aldrich), 4 mM MgCl2 (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-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 239g 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 (17,949g 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://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 < 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 re-

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**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 MIC80 value of AgNO3 (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 (**).
lease (Figs. 1 and 2) and proteins were recovered from cells as described, resolved by 2-D SDS PAGE and stained with colloidal Coomassie. A mild sonication step (2 × 10 s treatments) was employed to release protein present on the bacterial cell surface but this step had no effect on viability nor did it result in cell lysis. A total of 22 proteins (Fig. 3) were identified to be altered in abundance in the supernatant (Tables 1). Of these, 12 were involved in metabolism, six were involved in virulence and four in the stress response of *S. aureus*.

5. Discussion

While silver is now widely used clinically to inhibit the growth of microbes very little attention has been paid to the response of microbial cells to exposure to the Ag(I) ion and their attempt to withstand its effects. It has been demonstrated that metallic Ag(0) interacted with the peptidoglycan layer of *S. aureus* cells and led to the formation of pits (Mirzajani et al., 2011). Exposure to Ag(0) induced an increase in the abundance of muramic acid.
which is a major component of the peptidoglycan layer. Exposure of *E. coli* to Ag(0) nanoparticles caused an increase in the amount of reducing sugars and proteins released from the cells (Li et al., 2010). The exposure of *C. albicans* to Ag(0) nanoparticles promoted an antifungal effect through disruption of the cell membrane structure which was assessed by measuring the release of glucose and trehalose (Kim et al., 2009).

The results of the work presented here demonstrate that exposure to the Ag(I) ion affects the permeability of *S. aureus* cells as a significant increase in the release of protein from cells was observed. Protein release is indicative of increased permeability which can result in osmotic and/or oxidative stress within the cell. In addition, a variety of proteins associated with metabolism (12), virulence (6) and the stress response (4) were found to be released from cells when exposed to AgNO₃ for 2 h.

Some of the released proteins were associated with metabolism and included acetate kinase (spot 2) and succinyl CoA synthetase (spot 4) which were increased in abundance by 2.1-fold and 1.6-fold, respectively, following treatment with Ag(I) ions. The presence of phosphoglycerate mutase (spot 7) increased by 2.1-fold and this protein was also increased in expression following exposure of *E. coli* to Zn(II) ions (Sigdel et al., 2006). A number of proteins involved in translation demonstrated elevated levels of release from cells, including 30S ribosomal protein S2 (spot 5, 1.6-fold), 50S ribosomal protein L25/general stress protein (spot 6, 1.7-fold) and 50S ribosomal protein L31 type B (spot 12, 1.7-fold), suggesting that either the cells are responding to the Ag(I) ion by attempting to increase protein biosynthesis or these proteins are leaking from the cell due to altered cellular permeability.

The virulence of *S. aureus* can be attributed to the expression of adhesins and to the secretion of toxins (Bernardo et al., 2004). Alpha hemolysin (spot 16) is one of the main pore-forming toxins of *S. aureus*, allowing the efflux of cellular contents and the influx of Na⁺ and Ca²⁺ into the target cell and causing osmotic swelling and cell rupture (Dinges et al., 2000). Proteomic analysis identified a 2.1-fold decrease in the release of alpha hemolysin upon exposure of *S. aureus* to AgNO₃. It was also found that the abundance of secreted exoproteins, such as alpha- and beta-hemolysin, was reduced in *S. aureus* cells after exposure to the antibiotic linezolid (Bernardo et al., 2004). Leucocidin F and bifunctional autolysin were also reduced in expression in Ag(I)-treated *S. aureus* cells by −1.5 and −1.7-fold, respectively. There was an increase in the release of this protein in the exponential phase thus allowing the organism to establish an infection (Sanson et al., 2013). It was also found that this enzyme was required in the ability of the cell to tolerate oxidative stress (Singh and Moskovitz, 2003). Methionine sulphoxide reductase A (spot 22) is a transcription repressor protein (spot 20) which was increased in expression following exposure to the antibiotic, linezolid (Moskovitz, 2005). Methionine sulphoxide reductase A (spot 22) has been shown to play a key role in regulating the pathogenesis of bacterial cells by controlling a range of virulence factors (Balaban et al., 1998; Korem et al., 2005) and in tolerance to oxidative stress (Kiran and Balaban, 2009). It has been suggested that TRAP allows *S. aureus* to adhere to host cells when cells are at a low density and switches to express toxins in the late exponential phase thus allowing the organism to establish an infection (Balaban et al., 2001). The increase in the release of this protein suggests increased biosynthesis and that *S. aureus* may be responding to Ag(I) ion exposure by increasing the regulation of virulence factors.

Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the anti-oxidant defences which eliminate ROS before damage can occur. The abundance of a number of proteins involved in the stress response of *S. aureus* was increased upon Ag(I) ion treatment. These included a universal stress response protein (spot 19, +1.2), an alarmone shock protein (spot 21, +2.8), a transcription repressor protein (spot 22, +1.6) and methionine sulphoxide reductase A (spot 20, (MsrA) which showed a 2.9-fold increase in abundance. The Msr enzyme family comprises MsrA and MsrB which reduce S-MetO and R-MetO to methionine (Moskovitz, 2005). MsrA-specific methionine reductase activity in *S. aureus* plays a central role in the ability of the cell to tolerate oxidative stress (Singh and Moskovitz, 2003). Methionine sulphoxide reductase A has been shown to perform a critical role in the ability of *Leishmania major* to survive and replicate in macrophages thus highlighting a role for this enzyme in pathogenesis (Sanson et al., 2013). It was also found that this enzyme was
induced upon exposure of S. aureus to the antibiotic, oxacillin (Singh et al., 2001). The identification of elevated levels of these proteins released from S. aureus confirm that cells are experiencing stress as a result of Ag(I) ion exposure. This also suggests that the bacterium is attempting to mount a defence in response to the metal ion, which is an important attribute of a successful pathogen (Baker et al., 2010).

The results presented here indicate that exposure of S. aureus to Ag(I) ions induces the release of a broad range of proteins. A number of the released proteins are implicated in the ability of the bacterium to tolerate oxidative stress (e.g. TRAP) or play a role in tissue colonisation (e.g. methionine sulfoxide reductase A), and are potentially capable of stimulating a strong host immune response (Leitner et al., 2011). The widespread use of silver-containing ointments and plasters in the treatment of burn wounds raises the possibility of Ag(I) ions interacting with S. aureus at the wound site and altering its physiology. In addition, the change in the abundance of a range of proteins (TRAP, stress proteins, ABC transporter) may indicate an increase in the virulence of the pathogen (Leitner et al., 2011). The change in the abundance of a range of proteins (TRAP, stress proteins, ABC transporter) may indicate an increase in the virulence of the pathogen (Leitner et al., 2011).

Conflict of interest

The authors have no conflicts of interest to disclose.

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