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Exposure of *Staphylococcus aureus* to silver(I) induces a short term protective response

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Abstract The Ag(I) ion has well established anti-bacterial and antifungal properties. Exposure of *Staphylococcus aureus* to MIC_{80} $AgNO_3$ (3 µg/ml) lead to an increase in the activity of superoxide dismutase, glutathione reductase and catalase at 30 min but activity declined by 60 min. In addition, exposure of cells to this metal ion for 1 h lead to increased expression of a number of proteins such as elongation factors Ts, Tu and G, fructose-bisphosphate aldolase and triosephosphate isomerase but their expression declined following 4 h exposure. ATP binding cassette transporter protein and oligoendopeptidase F showed increased expression at 4 h. While Ag(I) is a potent antimicrobial agent this work demonstrates that *S. aureus* can mount a short-term protective response to exposure to the metal ion but that this is eventually overcome.

Keywords Antimicrobial · Silver(I) · *Staphylococcus* · Proteomics · Oxidative stress

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Introduction

Silver has well characterised antimicrobial properties and Ag(I) ions are active against a wide range of bacteria, fungi and viruses at concentrations of 10^{-9} to 10^{-6} M (Percival et al. 2005). The Ag(I) ion is the active agent in many healthcare products such as silver-coated catheters (Dasgupta 1994), wound dressings (Adams et al. 1999; Monafo and Freedman 1987) and creams for the treatment of burns (Wright et al. 1998). Clinical investigations into the anti-bacterial nature of silver-containing dressings have revealed the potent activity of these materials against *Pseudomonas aeruginosa* (Ulker et al. 2005; Olsen et al. 2000), while the effective anti-bacterial activity of the dressings was also demonstrated against methicillin resistant *Staphylococcus aureus* and vancomycin-resistant *Pseudomonas* species. Central venous catheters impregnated with silver metal particles substantially reduce the incidence of catheter-associated infections in paediatric patients (Carbon et al. 1999).

Although the mechanism(s) by which silver kills cells is not fully understood (Lok et al. 2006) there have been many suggestions regarding its mode of action. Ag(I) ions interact with thiol groups of the L-cysteine residue of proteins (Park et al. 2009; Cortese-Krott et al. 2009; Atiyeh et al. 2007; Castellano et al. 2007). This inactivates enzymatic functions involved in the respiratory chain such as NADH and succinate dehydrogenase which can impede electron transfer (Park et al. 2009). Another mechanism of

action involves the generation of reactive oxygen species (ROS) (Park et al. 2009; Cortese-Krott et al. 2009) which induces oxidative stress (Park et al. 2009) and leads to protein and DNA degradation (Park et al. 2009; Atiyeh et al. 2007; Castellano et al. 2007). Exposing the yeast *Candida albicans* to Ag(I) ions activates the Cap1p and Hog1p pathways as a consequence of oxidative and/or osmotic stress (Rowan et al. 2010).

Staphylococcus aureus, has the ability to survive in nutrient-limiting and stressful conditions (Clements et al. 1999), which contribute to its infectious nature. Approximately 30 % of healthy individuals carry *S. aureus* in the anterior nares (García-Lara et al. 2005), but once it contaminates a skin breach it can result in a variety of skin infections, ranging from superficial infections such as impetigo to more serious diseases such as toxic shock syndrome and sepsis (Pichon and Felden 2005). Treatment of *S. aureus* infections is complicated by the emergence of methicillin-resistant *S. aureus* (MRSA) and it is due to the increasing incidence of resistance to conventional antimicrobials that there has been a renewed interest in the use of Ag(I) as an antimicrobial agent (Cortese-Krott et al. 2009).

The aim of the work presented here was to ascertain the response of *S. aureus* to Ag(I) ions and to determine whether the bacterial cells were capable of mounting a response to withstand the antimicrobial effects of the metal cation.

Materials and methods

Organism and culture conditions

A clinical *S. aureus* urinary tract infection isolate was used. Cultures were grown on nutrient agar plates (Oxoid Ltd., Basingstoke, England) at 37 °C for 24 h and kept at 4 °C for short-term storage. Bacterial strains were cultured overnight in nutrient broth (Oxoid Ltd.) in an aerated conical flask in an orbital shaker at 37 °C and 200 rpm.

Susceptibility assay

Bacteria cultured to the stationary phase (OD_{600nm} of 2) overnight in nutrient broth, were diluted to an absorption value of 0.1 at 600 nm. To each well of a 96-well round-bottomed plate, 100 µl of fresh nutrient broth medium was added. From a stock solution of $AgNO_3$ (Aldrich) (200 µg/ml), 100 µl was added to each well and a serial dilution was carried out across the plate. Cells (100 µl) were added to each well and the plates were incubated at 37 °C for 24 h. The OD_{540nm} of the cultures was determined using a microplate reader (Bio-Tek, Synergy HT). All susceptibility assays were performed on three separate occasions. The MIC_{80} (Minimum Inhibitory Concentration) was defined as the concentration of compound that inhibited the growth of the bacteria by 80 %.

Enzymatic activity assays

Glutathione reductase activity was measured according to the method of Foster and Hess (1980). The reaction was followed by a reduction in the absorption at 340 nm on a Cary IE UV–Visible spectrophotometer, using an extinction coefficient value of 6.22 mM^{-1} cm^{-1} for NADPH. Superoxide dismutase (SOD) activity of silver Ag(I)-treated *S. aureus* cells was quantified using a SOD Assay kit (Sigma Aldrich Ltd.). The SOD activity was determined at 450 nm using a microplate reader. Catalase activity was measured using the method described by Larsen and White (1995). Protein extract (100 µl, 250 µg/ml) was added to 1.8 ml of 17 mM H_2O_2 in a sterile tube. The suspension was centrifuged at 10,000×*g* (Eppendorf centrifuge 5417R) for 1 min to stop the reaction. The supernatant was removed and dispensed into a quartz cuvette where the absorbance at 240 nm was read using a Beckman DU640 spectrophotometer. The blank consisted of 17 mM H_2O_2.

Whole cell protein extraction from *S. aureus*

Staphylococcus aureus cells were exposed to the Ag(I) ions and harvested using a Beckmann GS-6 centrifuge for 10 min at 1,814×*g*. The cell pellet was washed twice with sterile phosphate buffer saline (PBS) and re-suspended in 2 ml 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-piperazinedietansulphonic acid (PIPES) (Sigma-Aldrich)). Protease inhibitors (Leupeptin, Pepstatin A, Aprotinin and *N*-α-*p*-tosyl-L-lysine chloromethylketone hydrochloride (TLCK)), at a concentration of 10 µg/ml, were used. Acid-washed glass

beads (Sigma-Aldrich) were added and the suspension was vortexed on ice for 5 min. The resulting suspension was centrifuged in a Beckmann GS-6 Centrifuge for 5 min at 454×*g*, and the resulting supernatant was centrifuged on an Eppendorf 5417R centrifuge at 239×*g* for 4 min at 4 °C.

Separation of proteins by 2D SDS-PAGE

$AgNO_3$ at the MIC_{80} concentration (3 µg/ml) was added to cultures of *S. aureus* ($OD_{600nm} = 2.0$) and the cultures were then grown for pre-determined times. Cells were harvested and whole cell protein was extracted as described. Protein concentration was determined using the Bradford reagent (Bio-Rad, Munich, Germany). Protein was precipitated by the addition of ice-cold acetone. The precipitated protein was collected by centrifugation on an Eppendorf 5417R centrifuge (17,949×*g* for 30 min at 4 °C) allowed to air dry. Protein (300 µg) was separated by 2D SDS-PAGE as previously described (Kelly and Kavanagh 2010).

LC/MS mass spectrometric analysis of *S. aureus* proteins

Protein spots that exhibited altered intensities between control and Ag(I)-treated cells were excised, washed and trypsin digested as described (Shevchenko et al. 2006). Samples were analysed on a 6340 Ion Trap LC/MS spectrometer (Agilent Technologies) using bovine serum albumin 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 at $p < 0.05$.

Statistics

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

Results

Response of *S. aureus* to silver nitrate

Staphylococcus aureus cells were exposed to a range of concentrations of Ag(I) ions and the effect on growth was monitored. At a concentration of 0.78 µg/ml (4.6 µM) growth was reduced by 50 % and at 3 µg/ml (17.7 µM) growth was inhibited was 80 % (data not presented). Based on these results 3 µg/ml was chosen as the MIC_{80} value and this was used in subsequent experiments.

Changes in enzymatic activity following exposure to silver nitrate

The activity of SOD, GLR and catalase was measured in cells exposed to $AgNO_3$. When *S. aureus* cells were exposed to Ag(I) ions (17.7 µM) SOD activity increased after 30 and 60 min in comparison to the control (Fig. 1A).

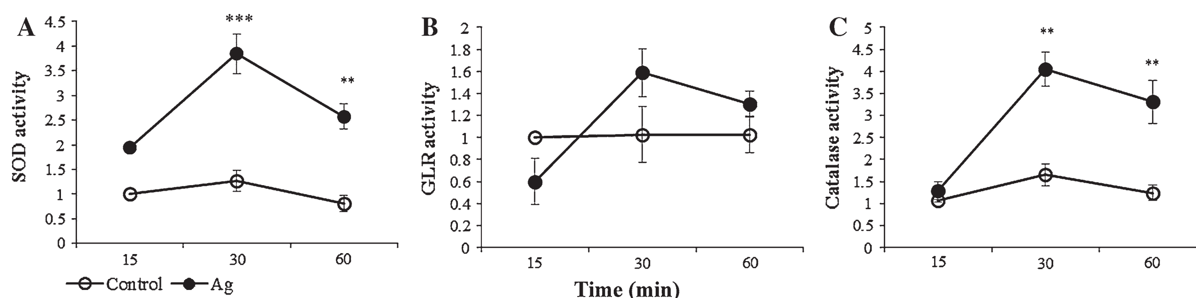

Fig. 1 Activity of **A** superoxide dismutase, **B** glutathione reductase and **C** catalase following exposure of *S. aureus* to Ag(I) ions. *S. aureus* cells were treated with the MIC_{80} value of Ag(I) ions for 15, 30 and 60 min, protein was extracted from the cells and enzymatic activity assessed as described. Differences in activity were deemed statistically significant at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$

For *S. aureus* cells exposed to Ag(I) ions there was a small but statistically insignificant increase in GLR activity at 30 and 60 min (Fig. 1B) compared to the control.

Catalase activity was measured in Ag(I)-treated *S. aureus* cells and compared to untreated control cells. A significant increase in activity was observed after 30 min and 60 min (Fig. 1C). These findings indicate that when *S. aureus* is exposed to Ag(I) ions at a concentration of 17.7 μM, there is an increase in the activity of three antioxidant enzymes which have the capacity to reduce the oxidative damage inflicted by exposure to the Ag(I) ions.

Changes in protein expression following exposure to silver nitrate

Cells were exposed to Ag(I) ions (17.7 μM) and whole cell protein was extracted, resolved by 2D electrophoresis (Fig. 2) and peptide spots were identified using LC/MS analysis (Table 1). A total of 11 proteins were observed to be altered in expression following exposure of *S. aureus* to the Ag(I) salt for 1 h. Eight of these proteins were identified as having putative roles in metabolism, two were involved in virulence and one was known to be associated with the stress response of *S. aureus*. Proteins such as elongation factors Ts, Tu and G (spots 236, 155 and 78, respectively), ornithine carbamoyltransferase (spot 237), dihydrolipoamide dehydrogenase (spot 147), fructose-bisphosphate aldolase (spot 314), 30S ribosomal protein S3 (spot 333) and triosephosphate isomerase (spot 340), play roles in metabolism. Proteins possibly associated with the virulence of *S. aureus* included an ATP binding cassette (ABC) transporter protein (spot 294) and also the phosphoserine 46 Hpr protein (spot 417). Oligoendopeptidase F (spot 111) is known to be involved in the stress response of *S. aureus*.

Discussion

An increase in SOD activity was observed following exposure of *S. aureus* cells to Ag(I) ions with the most significant increase in activity (3.8 ± 0.4-fold increase, $p < 0.001$) being observed at 30 min. Catalase activity increased at both the 30 and 60 min time points, with the most significant being at 30 min (4.1 ± 0.4-fold, $p < 0.01$). The elevation in enzymatic activity at 30 min may be evidence of an initial response to oxidative stress by the bacterium, and the

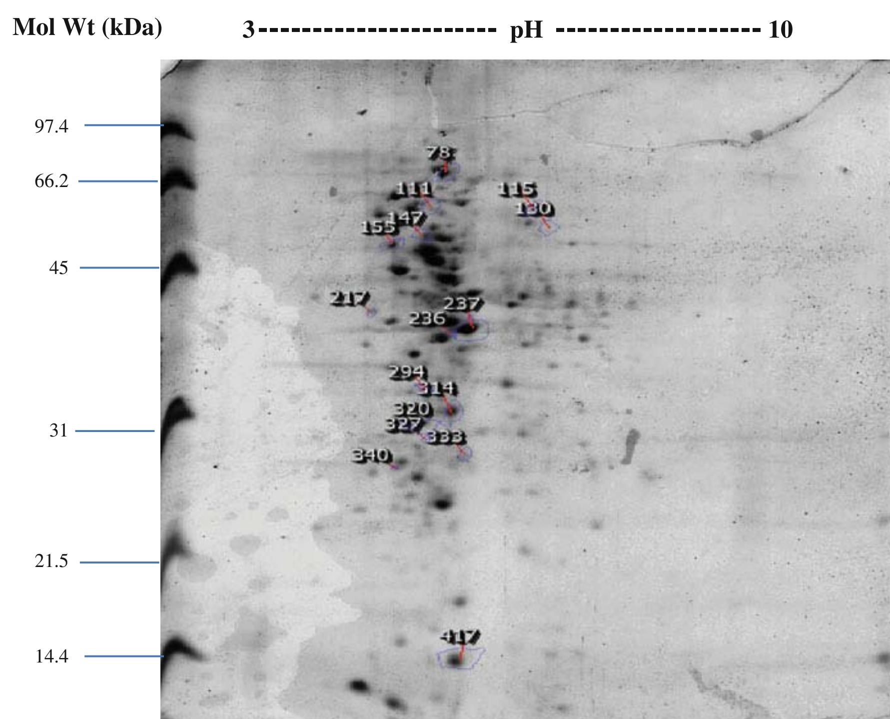

Fig. 2 Reference 2D gel for proteomic analysis. Progenesis SameSpot™ software reference image. Protein spots were analysed for fold changes in comparison to untreated cells, using Progenesis SameSpot™ software. Protein spots were extracted for identification using LC/MS

Table 1 Identity of peptides showing alteration in expression following exposure of *S. aureus* to Ag(I) ions for 1 or 4 h

Spot no.	Accession no.	Protein	Function	Score	% Coverage	Fold changes		Expectation (*p*) value
						1 h	4 h	
111	NP_814413	Oligoendopeptidase F	Proteolysis	260	10	1.1	1.5	8.76×10^{-5}
236	NP_816048	Elongation factor Ts	Protein biosynthesis	335	43	4.1	2.1	0.003
237	ZP_05425197	Ornithine carbamoyltransferase	Transferase activity	1126	74	1.7	−1.6	0.023
155	NP_371072	Elongation factor Tu	Protein biosynthesis	281	15	2.3	1.0	0.033
314	NP_814897	Fructose-bisphosphate aldolase	Fructose 1,6-bisphosphate metabolic process	387	33	1.4	−1.6	0.037
147	NP_815077	Dihydrolipoamide dehydrogenase	Flavin adenine dinucleotide binding	683	45	2.3	−1.1	0.039
333	NP_814010	30S ribosomal protein S3	Translation	221	23	2.0	−1.1	0.044
78	NP_371071	Elongation factor G	Protein biosynthesis	362	16	1.3	−3.2	0.048
340	NP_815638	Triosephosphate isomerase	Glycolysis	324	39	1.9	−1.3	0.055
294	YP_002633557	ABC transporter	ATP-binding	404	25	1.5	2.7	0.057
417	1FU0_A	The phosphoserine 46 Hpr	Phosphotransferase system	98	13	1.7	1.0	0.059

decline in activity by 60 min may indicate a loss in cell function or death.

Proteomic analysis revealed an increase in expression of a range of proteins following exposure of *S. aureus* cells to Ag(I) ions however a number of these proteins had declined in expression by 4 h. At the 1 h time point, there was an increase in the expression of elongation factors Ts, Tu and G by 4.1-, 2.3- and 1.3-fold, respectively, but these subsequently decreased in relative expression by 4 h. Kaakoush et al. (2008) also found that the elongation factors Tu and G of *Campylobacter jejuni* were upregulated as a result of exposure to Cd(II) ions. The present results using Ag(I) ions are interesting since elongation factors are required for extending the polypeptide chain in protein biosynthesis which is essential for microbial growth and development (Berisio et al. 2010), and suggests that additional protein biosynthesis occurs in response to metal ion exposure (Kaakoush et al. 2008).

Fructose-bisphosphate aldolase and triosephosphate isomerase in the Ag(I)-treated *S. aureus* cells were found to be increased in expression at 1 h and decreased in expression by 4 h. Fructose bisphosphate aldolase has previously been reported to be increased in expression in caspofungin-treated *C. albicans* cells, where it was postulated to be contributing to a stronger immune response (Kelly and Kavanagh 2010). With the $AgNO_3$-treated *S. aureus* cells, a third protein, dihydrolipoamide dehydrogenase, was increased in expression by 2.3-fold at 1 h but this decreased to −1.1-fold at 4 h.

Proteins involved in the virulence of *S. aureus* included an ABC transporter protein (spot 294) and phosphoserine 46 Hpr (spot 417). *S. aureus* cells responded to $AgNO_3$ exposure by significantly increasing the expression of the ABC transporter at 1 and 4 h (spot 294; 1.5- and 2.7-fold, respectively), suggesting an increase in the efflux of xenobiotics produced as a result of exposure to the Ag(I) ion. Phosphoserine 46 HPr plays an important role in the carbon catabolite repression system (Gorke and Stulke 2008) and its expression was increased by 1.7-fold at 1 h before returning to basal level at 4 h.

Oligoendopeptidase F (spot 111), which is involved in proteolysis (Jenal and Hengge-Aronis 2003), also increased in expression by 1.5-fold at 4 h. This protein is thought to contribute to the virulence of this organism since Group A *Streptococci* has been shown to evade the host immune response by disrupting the recruitment of phagocytic cells through the action of a serine endopeptidase (Voyich et al. 2004).

The results presented here indicate that exposure of *S. aureus* to the MIC_{80} value of $AgNO_3$ leads to a transient increase in the activity of SOD, GLR and catalase after 30 min and an increase in expression of a range of proteins having diverse functions after 1 h exposure. By 60 min, the level of enzymatic activity has declined, and by 4 h the expression of many of the proteins has returned to basal levels or below. The significance of these results is that short term exposure to sub-lethal concentrations of $AgNO_3$ induces the bacterium to mount a response to counteract the effects of Ag(I) ions (e.g. increased enzymatic activity, elevated expression of elongation factors). Prolonged exposure to $AgNO_3$ leads to reduced enzymatic activity and protein expression indicating that the Ag(I) ions can overwhelm the cell and cause its demise. Initially, *S. aureus* actively attempts to limit the damage imposed by exposure to the Ag(I) ion.

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