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Use of bioreporters and deletion mutants reveals ionic silver and ROS to be equally important in silver nanotoxicity

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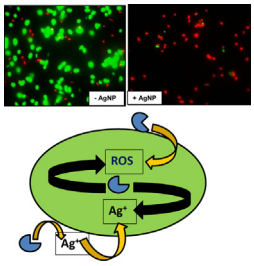
HIGHLIGHTS

- Novel bioreporter was developed and employed to investigate silver nanotoxicity.
- Response of deletion mutant strains for oxidative stress and metal efflux gene to AgNp suggest that both ROS and ionic silver are important.
- Oxidative stress plays an important role in causing cell death.
- Presence of chloride ions does not completely eliminate silver nanotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

The mechanism of antibacterial action of silver nanoparticles (AgNp) was investigated by employing a combination of microbiology and geochemical approaches to contribute to the realistic assessment of nanotoxicity. Our studies showed that suspending AgNp in media with different levels of chloride relevant to environmental conditions produced low levels of ionic silver thereby suggesting that dissolution of silver ions from nanoparticulate surface could not be the sole mechanism of toxicity. An *Escherichia coli* based bioreporter strain responsive to silver ions together with mutant strains of *E. coli* lacking specific protective systems were tested against AgNp. Deletion mutants lacking silver ion efflux systems and resistance mechanisms against oxidative stress showed an increased sensitivity to AgNp. However, the bioreporter did not respond to silver nanoparticles. Our results suggest that oxidative stress is a major toxicity mechanism and that this is at least partially associated with ionic silver, but that bulk dissolution of silver into the medium is not sufficient to account for the observed effects. Chloride ions do not appear to offer significant protection, indicating that chloride in receiving waters will not necessarily protect environmental bacteria from the toxic effects of nanoparticles in effluents.

1. Introduction

Engineered metal-based nanoparticles (NP) such as silver, zinc oxide and titanium dioxide are toxic to bacteria and other microorganisms because of the enhanced reactivity arising from their small size [1,2]. In addition to their chemical properties, variables including the presence of capping agents, shape of the nanoparticles, oxidation state and pH affect toxicity [3–5]. Recent studies indicate that oxidative stress and dissolved ionic species play an important

Abbreviations: NP, nanoparticle; ROS, reactive oxygen species; FSU, relative fluorescence unit; M9RC, M9 medium with reduced chlorides; OD600, optical density at 600 nm; EYP, enhanced yellow fluorescent protein; CFU, colony forming unit; ATP, adenosine triphosphate.

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role in nanoparticle toxicity, which may also involve membrane damage and protein oxidation [6–11]. However, the overall picture remains ambiguous, especially while attempting to extrapolate from laboratory experiments to the natural environment where physicochemical transformations of nanoparticles can take place [12,13]. The extent and nature of the damage varies with the type of nanomaterial and exposure conditions. For instance, the presence of ligands such as chloride and phosphate and changes in pH, can influence the speciation of dissolved nanoparticles and hence further complicate mechanistic interpretations [14,15].

Toxicity studies using silver nanoparticles have generated much debate about the toxicity mechanisms in the recent past. Some studies suggested that AgNp operated via particle specific effects [16,17], including oxidative stress [7] and ionic toxicity. Xiu et al. [18] questioned particle-specific effects and by extension that oxidative stress may also arise from ionic silver [19,20]. However, this view is being increasingly challenged in more recent studies as we develop a better understanding of silver speciation in different media [14]. Existing studies have largely relied on assessing cell viability, with oxidative stress being detected using fluorescent probes that at times provide unreliable data [21]. Microarray studies conducted on *Escherichia coli* using silver and polystyrene nanoparticles indicate that multiple genetic pathways are up-regulated on exposure to nanoparticles [22,23]. A systematic investigation of the interaction of bacteria with engineered nanoparticles using stress responsive and metal ion responsive genes in the bacteria under investigation could provide better insight into the toxicity mechanisms.

E. coli is a model bacterium with well-understood stress resistance mechanisms. All of the non-essential genes in *E. coli* have been individually mutated, and mutant strains are available from the KEIO collection [24]. Furthermore, bioreporter strains can be developed in which known stress-responsive promoters are linked to reporter genes.

In this study, we have used mutated strains of *E. coli* lacking specific protective mechanisms to re-assess the relative roles of ionic and nanoparticulate silver on toxicity to bacteria. We have also used a bioreporter to test the bioavailability of ionic silver in a medium containing chloride at concentrations in the range found in urban freshwater and upper estuarine bodies (0–10000 mg/L up to the mesohaline) [25], which are the first receptors of NP discharges and always contain some chloride from road run-off [12,26,27]. Based on recent predictions that ionic silver precipitates as silver chloride in freshwaters [28], it was hypothesised that ionic silver would have minimal involvement in nanoparticle toxicity.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains used in this study are listed in Table 1. Deletion strains were obtained from the KEIO collection via the Yale Genetic Stock Centre [24]. The triple deletion strain LE106, lacking *katG, katE* and *ahpF*, was kindly provided by Prof. James Imlay, University of Iowa [29]. A biosensor responsive to copper and silver ions was developed by cloning the promoter of *copA* [30–32] in BioBrick RFC10 format [33,34] in vector pSB1C3 (Registry of Standard Biological Parts). The following primers were designed to amplify the promoter:

5′-CCTTGAATTCGCGGCCGCTTCTAGAGTGAAATTGGGTGTAAGC-3′

5′-AAGGCTGCAGCGGCCGCTACTAGTACTGTGATAAAGGTTAAA-CC-3′

A reporter gene, enhanced yellow fluorescent protein (EYFP, BioBrick BBa_E0040), was then inserted downstream of the promoter to generate the final reporter construct, designated pSB1C3/P*copA* + EYFP.

2.2. Reagents

Components of the growth medium, catalase, and sodium citrate-stabilized silver nanoparticles were obtained from Sigma–Aldrich. The LIVE–DEAD cell viability kit was procured from Invitrogen. Phosphate buffered saline (PBS) was made by adding 8 g/L NaCl, 0.2 g/L KCl and 1.44 g/L Na_2HPO_4 in deionized water. The pH was adjusted to 7.4 and the solution sterilized by autoclaving. Catalase (Sigma catalogue number C9322) at 5 mg/ml was suspended in PBS and added to the cultures to achieve a final concentration of 100 U/ml (30 μg/ml).

2.3. Growth conditions and assays

Exposure studies used M9 medium, made by diluting a 4× stock solution containing 64 g/L $Na_2HPO_4{\cdot}7H_2O$, 15 g/L KH_2PO_4, 5 g/L NH_4Cl and 2.5 g/L NaCl. This was supplemented with 0.011 g/L calcium chloride, 1.204 g/L magnesium sulphate, 2 g/L Casamino acids, 0.34 g/L thiamine hydrochloride and 0.4% w/v glycerol as a carbon source. This medium contained approximately 1225 mg/L chloride ions. Control tests for silver chloride precipitation were conducted by using M9RC (Reduced Chloride) medium consisting of the same medium modified by replacing sodium chloride with sodium nitrate and ammonium chloride with ammonium sulphate

Table 1
Strains of *E. coli* used for exposure assay in this work.

Bacterial strain	Genotype	Details
E.coli JM109/pSB1C3/P*copA* + EYFP	Lab strain transformed with plasmid pSB1C3-PcopA_YFP. It forms yellow fluorescence protein on induction with silver ions	This work
MG1655		Wild type *E. coli*, parent strain of LE106
LE106		Triple deletion mutant strain Δ*katG*/Δ*katE*/Δ*ahpF*
BW25113		Parent strain of KEIO strains.
JW0473-3	Δ*copA*767::*kan*	Deletion of copper efflux pump
JW0476-1	Δ*cueR*770::*kan*	Deletion of regulator of *copA*
JW0564-1	Δ*cusA*784::*kan*	Deletion of cation transporter
JW0563-2	Δ*cusB*783::*kan*	Deletion of copper/silver efflux membrane fusion protein
JW0562-1	Δ*cusF*782::*kan*	Deletion of copper/silver efflux system protein
JW0560-1	Δ*cusR*780::*kan*	Deletion of DNA binding transcriptional activator of *cusABF*
JW3914-1	Δ*katG*729::*kan*	Deletion of catalase
JW1721-1	Δ*katE*731::*kan*	Deletion of catalase
JW3879-1	Δ*sodA*768::*kan*	Deletion of superoxide dismutase A
JW1648-1	Δ*sodB*734::*kan*	Deletion of superoxide dismutase B
JW0005-1	Δ*yaaA*726::*kan*	Deletion of peroxidase
JW0422-1	Δ*cyoA*789::*kan*	Deletion of cytochrome bo terminal oxidase subunit
JW0421-1	Δ*cyoB*788::*kan*	Deletion of component of cytochrome bo terminal oxidase
JW0723-2	Δ*cydB*782::*kan*	Deletion of cytochrome *bd*-I terminal oxidase subunit

at equivalent molar concentrations, giving approximately 7.1 mg/L chloride ions. Plasmids based on pSB1C3 were maintained by addition of chloramphenicol (40 μg/ml). Cultures were grown at 37 °C with shaking at 150 rpm. Cell viability was assessed by serial dilution, and cultures were plated on LB-agar. Percentage cell viability for a bacterial strain was calculated by normalizing the value for a treatment condition to its own control without any silver nanoparticle or silver nitrate, thus accounting for differences in growth rates between mutants. ATP assay was performed by using the Bactiter Glo Kit from Promega according to the manufacturer's protocol. Multimode reader (BS040271) from Turner Biosystems was used for both the ATP assays (luminescence mode) and measuring response of biosensor cells (fluorescence mode).

2.4. Nanoparticle source and characterization

A 10 nm silver nanoparticle dispersion at 20 mg/L concentration was obtained from Sigma–Aldrich. Size characterization was carried out in the presence of water and M9 medium by dynamic light scattering (DLS) using a ZETA PALS 90 sub-micron analyser (Brookhaven Instruments Corporation Holtsville, NY, USA). The samples were sonicated (230 V, 50 Hz) for 5 min prior to use. The data were collected in triplicate at a temperature of 25 °C. The extent of nanoparticle dissolution was determined after suspending known amounts (7 mg/L) of nanoparticles in deionized water, M9 medium and modified M9 medium in which all chlorides were replaced with nitrate and sulphate at the same concentration as described above. These samples were incubated with rotary shaking (200 rpm) at 37 °C for 2 h, the same time as the exposure tests. The supernatant was recovered by centrifugation at 3000 × *g* using Amicon filters (3000 MWCO, Millipore) for 60 min at 10 °C. It was then acidified by addition of 2% (v/v) nitric acid and analyzed using ICP–OES (Optima 5300DV, PerkinElmer) [35]. Solutions of silver nitrate (4 mg/L ionic silver equivalent) were incubated in parallel to check recovery of dissolved silver.

2.5. Exposure experiments

For KEIO mutant strains, bacteria were grown overnight in minimal medium with kanamycin (50 μg/mL) to prevent contamination. The optical density at 600 nm (OD600) was adjusted to 0.2 by diluting with fresh medium. Nanoparticles were added to final concentrations of 5.5 and 7 mg/L. The cultures were incubated at 37 °C for 120 min on a rotary shaker (150 rpm) in the dark. For dissolved silver toxicity studies, the procedure was the same except that silver (ionic) in the form of silver nitrate ($AgNO_3$) was added at final concentrations of 3.5 and 8.5 mg/L (equivalent total silver being 2 and 5.4 mg/L, respectively). Viability and luminescence assays were conducted as described above. For one set of experiments, modified M9 medium was made by replacing chlorides with sulphate and nitrate as described above, in order to investigate possible changes in toxicity in the presence on ligands other than chloride. Eh of the suspensions was not measured but it was assumed that they remained aerobic during the 2 h incubation due to air headspace in the vials.

Since the growth rates of the different strains/mutants were different, direct comparison based on absolute CFU was not feasible. Instead, cell viability for each strain/mutant was calculated as a percentage by normalizing the mean value for each treatment condition (e.g., 7 mg/L nanoparticle addition) to its own control without any silver nanoparticles or silver nitrate, thus accounting for differences in growth rates. This automatically assigns a survival of 100% to the control (no nanoparticle) incubation, thereby allowing a pairwise comparison between the control and each treatment using the student *t*-test. Survival rates of different strains at a given nanoparticle treatment were then compared using ANOVA

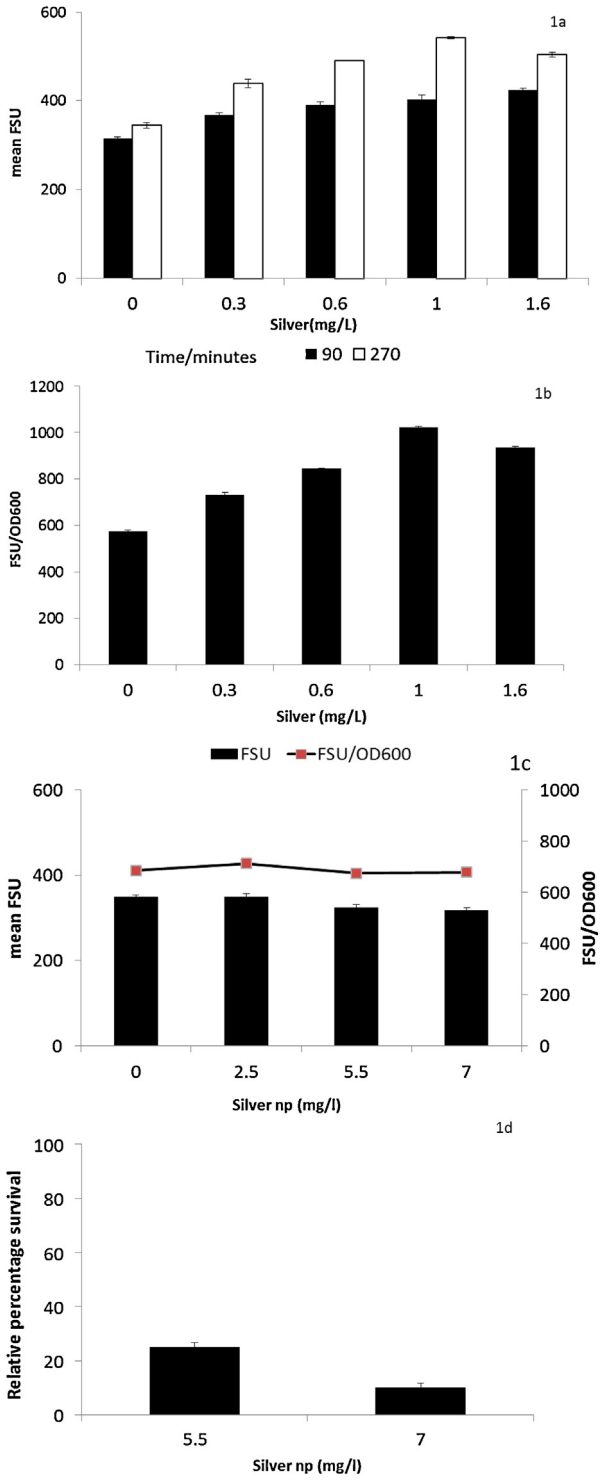

Fig. 1. Graphs showing relative fluorescence units (FSU, a–d) and cell survival (e) for the silver ion bioreporter. (a) Fluorescence response to $AgNO_3$ at 90 and 270 min incubation. (b) Response normalized to OD at 270 min, showing a maximum response at 1 mg/L and decline at higher concentrations as cell survival is compromised. (c) Response (fluorescence units, FSU) to silver nanoparticles (AgNp) after 270 min of incubation. Secondary axis shows the fluorescence values normalized to OD600 at time 270 min. (d) Cell viability after nanoparticle exposure normalized to control without $AgNO_3$ or nanoparticles, suggesting that lack of bioreporter induction in the presence of nanoparticles is due to high cell mortality.

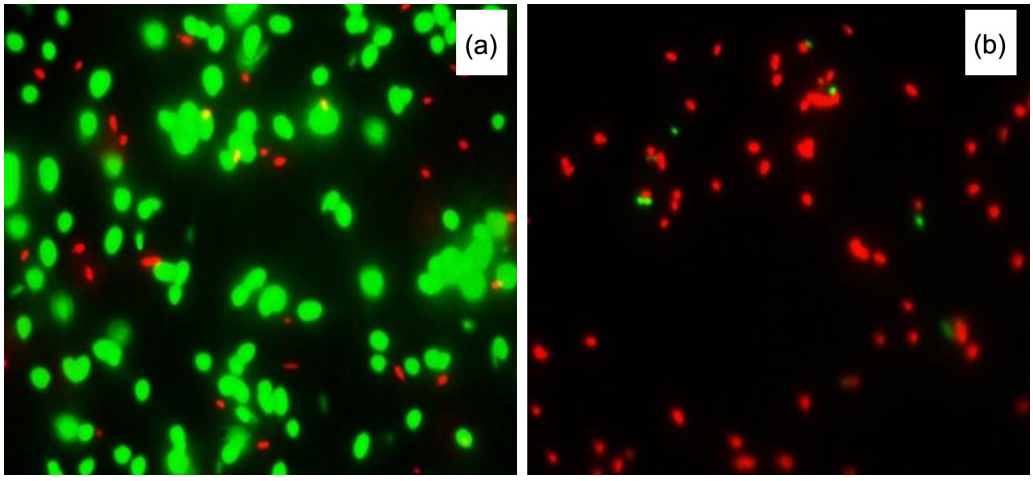

Fig. 2. Live Dead images of the bioreporter cells before (a) and after treatment with silver nanoparticles (b). The high mortality rate upon exposure to nanoparticles occurs despite low ionic silver concentrations in nanoparticle supernatants, suggesting that lack of bioreporter response is due to cell mortality and further that ionic silver is not the only nanotoxicity mechanism.

followed by Tukey's post hoc honestly significance difference (HSD) test on treatment means.

3. Results

3.1. Assessment of silver bioavailability using a bioreporter

A biosensor (pSB1C3/P*copA* + EYFP) that responds to ionic silver was developed by using the CueR-regulated promoter of *copA* as discussed earlier. The biosensor responds to increasing concentration of silver nitrate with total added silver in the range 0.3–1.6 mg/L, after which it showed a drop in fluorescence (Fig. 1a and b), most likely as cell viability declines, hence quenching fluorescence. When the biosensor was tested with silver nanoparticles, there was no significant induction (Fig. 1c), suggesting either that there was insufficient ionic silver in the cytoplasm to induce the biosensor, or high cell mortality due to mechanisms other than ionic toxicity. Supernatants recovered from medium containing nanoparticles failed to induce fluorescence in biosensor cells (Fig. SI.1), consistent with low ionic silver concentrations. In contrast, exposure to AgNp adversely affected cell viability based on growth, as shown in Fig. 1d, and also confirmed using LIVE/DEAD staining and fluorescence imaging (Fig. 2). This suggests that the nanoparticles were toxic irrespective of the bioavailable Ag^+, and is consistent with toxicity mechanisms unrelated to the presence of ionic silver.

3.2. Impact of AgNp on cell viability of strains deficient in genes for oxidative stress defense and respiratory subunits

Numerous studies, using various biochemical assays, have reported that oxidative stress plays a major role in nanoparticle toxicity. *E. coli* KEIO strains with mutations in important oxidative-stress resistance components of the *oxyR* and *soxR* regulons as shown in Table 1 were tested. Most of these strains showed greater sensitivity to silver nanoparticles relative to the parent strain (Fig. 3a). Significantly, they also showed greater sensitivity than the parent strain in the presence of silver nitrate (Fig. SI.2), suggesting either the presence of ionic silver even in these chloride-containing growth media or that the AgCl precipitate is still capable of inducing toxicity *via* oxidative stress or other mechanisms such as membrane damage. *E. coli* strain LE106, with three major catalases and peroxidases, *KatG*, *KatE* and *ahpF*, deleted, demonstrated an enhanced susceptibility to silver nanoparticles (Fig. 3b) as com-

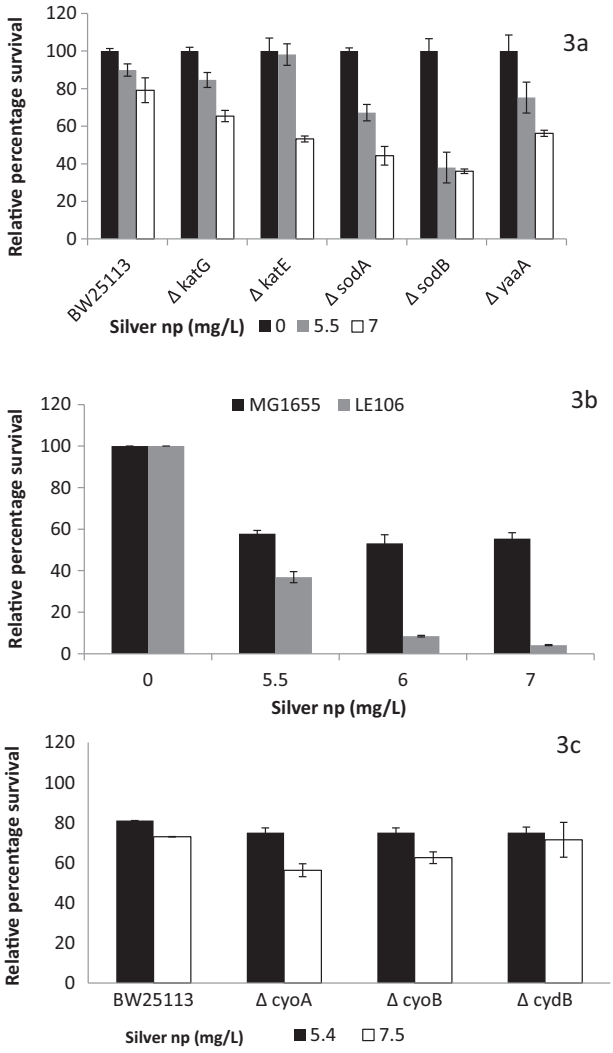

Fig. 3. Graphs showing viability of different deletion mutants upon exposure to silver nanoparticles. (a) Deletion mutants for oxyR and SoxR regulon; (b) triple deletion (Δ*katG*/Δ*katE*/Δ*ahpF*) strain LE106, (c) deletion mutants lacking cytochrome oxidase subunits. The percentage cell viability for each nanoparticle treatments is determined by normalizing CFU/mL values of the treated samples to its own control without any nanoparticles.

pared to the control strain MG1655. The mutant strains were also sensitive to silver nitrate.

Aerobic respiration in *E. coli* requires the cytochrome oxidase genes encoded by the *cyo* (cytochrome bo) and *cyd* (cytochrome bd) operons. It has been reported that *cyoA*, encoding a subunit of terminal oxidase cytochrome bo, is upregulated on exposure to silver nanoparticles [22]. In survival assays, *cyoA* and *cyoB* mutants showed increased sensitivity to nanoparticles (Fig. 3c). This may reflect a specific effect, or may be due to reduced energy availability in such mutant strains. The deletion of *cydB* had little or no impact on cell viability. Under the aerated conditions used, it is plausible that cytochrome bo was the major respiratory system expressed.

To investigate if these effects were due to oxidative stress, catalase (30 μg/ml final concentration) was added during nanoparticle exposure. Catalase helps decomposes hydrogen peroxide. Addition of catalase led to an increase in cell viability as determined by colony counts (Fig. 4) and total ATP content (SI.3). The protective effect was much more pronounced in the mutant strains ($p = 0.03$ and $p = 0.01$ for Δ*katG* and Δ*katE,* respectively at 7 mg/L silver nanoparticles) than in the parent strain, suggesting that survival recovery in deletion mutants was not solely due to thiolate complexation of ionic silver. To confirm this, the experiment was repeated using heat-denatured catalase, and an inactive control protein (100 μg/mL ovalbumin). Both treatments also produced a significant increase in cell viability, but only at a considerably higher concentration than with active catalase. On testing nanoparticle stability in the presence of catalase and albumin, it was found that protein concentrations greater than 80 μg/mL led to increase in average diameter of silver nanoparticles [36]. The presence of catalase at the concentration used in the cell viability assay, however, did not produce such aggregation, suggesting that the protective effect observed was due to enzyme activity.

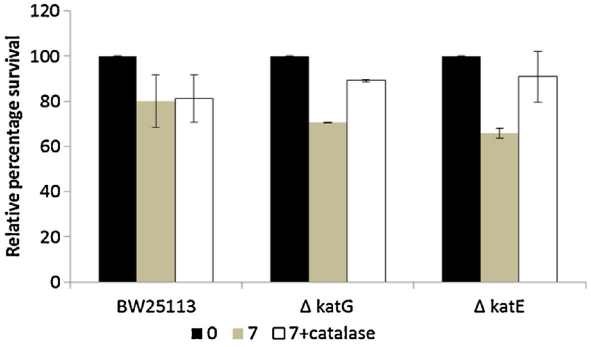

Fig. 4. Graphs showing viability of selected oxidative stress deletion mutants upon exposure to nanoparticles following addition of catalase. The numbers in the legend in Fig. 4 represent concentration nanoparticles in mg/L.

3.3. Effect of deleting genes responsible for efflux of silver ions in bacteria

To further explore the role of ionic silver, deletion mutants lacking genes encoding for subunits that form the efflux pumps for copper and silver ions (Δ*cueR* and Δ*cusF*) were used. Limited tests with $AgNO_3$ on two of these strains, along with the control strain BW25113 showed that these strains were more sensitive

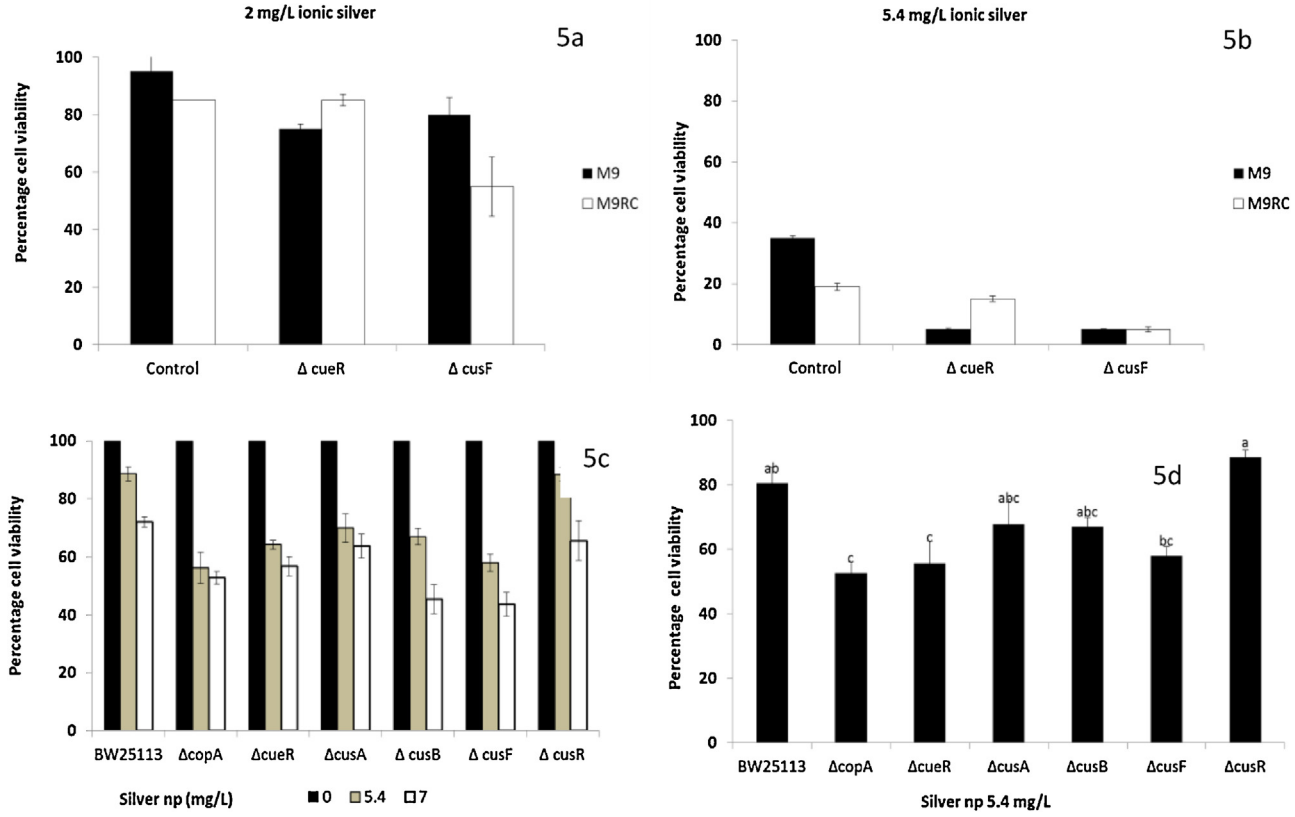

Fig. 5. Graphs showing survival of mutants lacking metal efflux genes in the presence of (a) 2 mg/L silver (3.5 mg/L $AgNO_3$) in M9 and M9RC (reduced chloride), (b) 5.4 mg/L silver (8.5 mg/L $AgNO_3$) in M9 medium and M9RC. Data for each strain is normalised to CFU at 0 mg/L $AgNO_3$ to account for differences in growth rate among strains. Deletion mutants are generally more susceptible to ionic silver but presence of chloride does not have significant effects. (c) Response of deletion mutants for Cu/Ag to silver nanoparticles in M9, confirming that all mutants are more susceptible relative to control strain. (d) Statistical analysis of the data at 7 mg/L nanoparticle exposure, showing that Δ*cueR* and Δ*cusF* are significantly more susceptible than the other strains. The similar letters above bar indicate the results are statistically not significant for those points.

Table 2
Dissolved silver levels for different condition (mg/L).

Medium with AgNp/$AgNO_3$ (mg/L)	Deionized water + silver np (7)	M9 + AgNp (7)	M9RC + AgNP (7)	M9 + $AgNO_3$ (4)	M9RC + $AgNO_3$ (4)
Residual Ag + after incubation and ultra centrifugation	0.09	0.014	0.056	0.12	0.09

than the control strain (Fig. 5a and b) but that changing chloride concentration did not significantly affect the outcome. This observation suggests either the levels of ionic silver in the exposure medium were sufficiently high to contribute to toxicity or that the colloidal/particulate forms of Ag formed by precipitation in medium also contributed to total toxicity. Indeed, when metal efflux deletion mutants (Table 1) were tested for sensitivity to silver nanoparticles, all but one (Δ*cusR*, lacking a regulatory gene but with structural genes intact) showed a significant drop in cell viability relative to the control strain (Fig. 5c). The differences in cell viability of these strains on exposure to AgNp was analyzed by the Tukey test and found to be significant as shown in Fig. 5d. However, in assays with both metal efflux and oxidative stress deletion strains, the control strain was also found to be sensitive at high concentrations of $AgNO_3$. Considering that chloride is in excess relative to ionic/nanoparticulate silver in the exposure medium, the only mechanisms for maintaining high ionic silver concentrations are either slow AgCl precipitation kinetics or the possibility that bacteria act as a continual sink that promotes continuous dissolution of the AgCl precipitate and silver nanoparticles [28].

3.4. Total dissolved silver in exposure media

Since the biosensor and deletion mutants suggest different mechanisms of toxicity, with the biosensor in particular suggesting that ionic silver is not involved, nanoparticle suspensions were characterized for size distribution and in particular for dissolved silver, the latter being operationally defined as silver in the filtrate after centrifugal filtration using 3000 MWCO Amicon filters [35]. Particle size analysis showed that the nanoparticles maintained their nominal size when dispersed in de-ionized water (9 ± 2 nm) and in M9 exposure medium (12 ±3 nm). The dissolution of silver nanoparticles was investigated by incubating AgNp (7 mg/L) for 2 h (equivalent to the exposure time) in deionized water, M9 medium and M9RC medium in which chloride was replaced by sulphate and nitrate. The dissolved silver levels for each condition (mg/L) have been shown in Table 2. After 2 h of incubation, medium containing 7 mg/L AgNp, produced total dissolved silver as follows; 0.09 mg/L (1%) in deionized water, 0.014 mg/L (0.2%) in M9 and 0.056 mg/L (0.8%) in M9RC. In comparison, incubation of 4 mg/L $AgNO_3$ in M9 and M9RC gave 0.12 mg/L (3%) and 0.09 mg/L (2%), respectively.

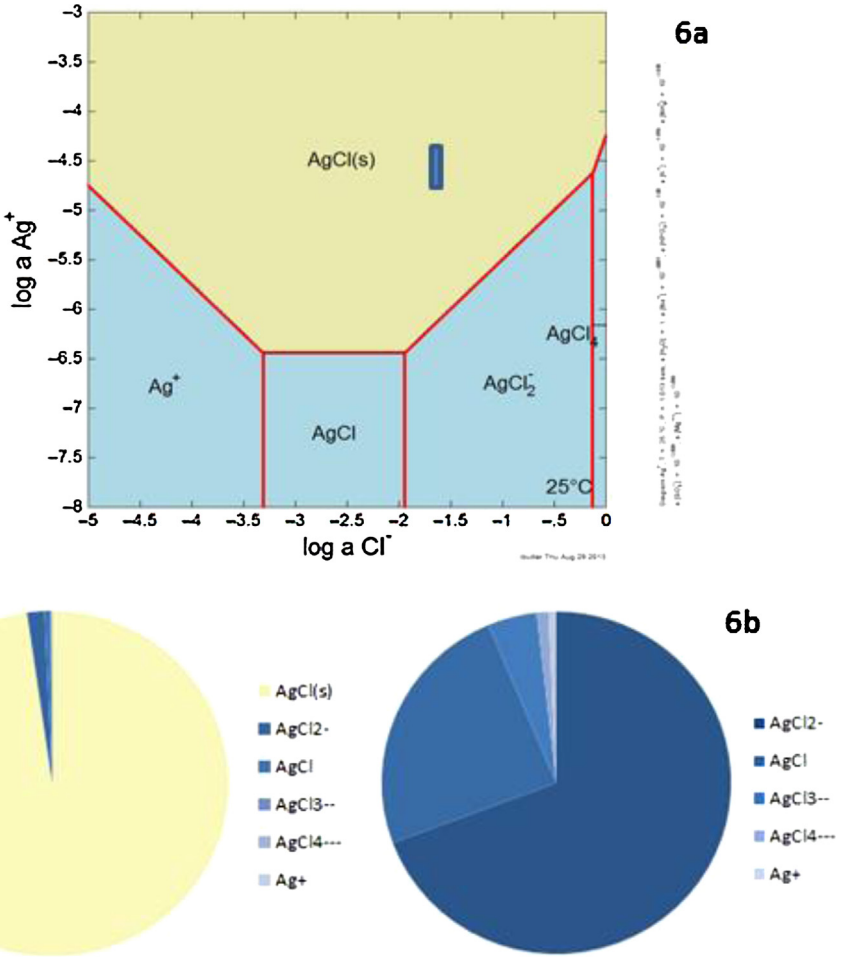

Fig. 6. Speciation of silver in exposure media as modelled using the Geochemists' Workbench v7.0.6 React code for 7 mg/L total silver (both ionic and nanoparticulate). (a) Activity–activity diagram showing that the media are supersaturated with silver chloride based on the range of dissolved silver and chloride defined by the blue bar in the AgCl(s) field. (b) Distribution of all species in the medium, showing that ~97% of the silver is precipitated as silver chloride and (c) distribution of dissolved species after AgCl precipitation. Tetra-aqua cationic silver represents less than 1% of the total dissolved silver.

It was considerably higher than the concentrations observed with silver nanoparticles, but still indicative of extensive precipitation.

4. Discussion

Silver nanoparticles are toxic to bacteria, and recent studies propose that release of ionic silver from the nanoparticulate surface is the major factor contributing to toxicity [9,26,36–38]. In this study, the biosensor P*copA* + EYfp responded to ionic silver ($AgNO_3$) and not to AgNP, although exposure to NPs affected cell viability significantly. Nanoparticle dissolution experiments showed that less than 2% of total silver added as nanoparticles was available in a dissolved form in water and it was further decreased in M9 medium. Similarly, only 3% of the added ionic silver remained in solution in M9. These results are consistent with precipitation of silver chloride and are confirmed by equilibrium speciation modelling using Geochemists' Workbench [37]. The model confirmed that AgCl was the thermodynamically stable phase (Fig. 6a). Furthermore, speciation of the residual dissolved silver after precipitation of the AgCl predicts that less than 1% ($\sim 8 \times 10^{-9}$ mol/L) exists as ionic silver (tetra-aqua cation or ionic silver), with the rest being neutral AgCl (~29%), $AgCl_2^-$ (~68%) and $AgCl_3^{2-}$ (~2%) complexes (Fig. 6b).

Previous reports have described up to a 20% loss in viability in biofilms in the presence of comparable (ionic) silver concentrations of 0.001 mg/L [38]. However, our data for the biosensor, oxidative stress and metal efflux deletion mutants are consistent in revealing much higher mortality than can be attributed to ionic silver alone. Thus, dissolution of silver ions from nanoparticles is not the only explanation for the observed toxicity. Besides ionic silver, complexed silver species such as $AgCl_{2\ (aq)}^-$ can also act as a labile source of silver ions to bacteria via thermodynamic equilibration between the bulk solution and the cell surface. This observation is consistent with the Biotic Ligand Model [39]. In turn, any uptake by cells will drive a constant and steady flux from nanoparticles, potentially leading to locally elevated concentrations in the vicinity of the nanoparticles [40]. Indeed in our assays, mutant strains lacking efflux pumps capable of exporting silver ions showed increased sensitivity to silver nanoparticles. All these observations point to an important role for ionic silver.

Nevertheless, mutants lacking major oxidative stress response systems also showed enhanced sensitivity to silver, consistent with previous reports [9,19,41]. In this study, the *sodB* deletion [41] mutant was found to be most susceptible. In previous reports, *sod* deletion strains were found to be highly sensitive to various types of metal nanoparticles. For instance, *sodA* and *sodM* deletion mutants of *Staphylococcus aureus* are highly sensitive to zinc oxide nanoparticles [42]. This suggests that superoxide radicals may be one of the major reactive oxygen species (ROS) being formed during exposure to silver nanoparticles. The interaction between nanoparticles and oxidative stress is not straightforward; silver ions could themselves act as oxidizing agents, nanoparticle surfaces may act as catalytic surfaces for ROS formation, and furthermore, production of ROS due to general stress might oxidize nanoparticles to release more silver ions [41]. Thus production of reactive oxygen species is likely to be as important as ionic silver with the two mechanisms operating synergistically.

5. Conclusions

Recent studies have focused on resolving the exact mechanism by which silver nanotoxicity to bacteria operates and predominantly conclude that toxicity occurs via nanoparticles releasing ionic silver. The implication is that in most natural environments, particularly those likely to be first level receptors of nanoparticle release (freshwaters, urban ponds etc.), the presence of chloride coupled with the predicted precipitation of AgCl in such media will ameliorate silver nanotoxicity. Our results demonstrate that ionic silver is an important toxicity mechanism even in chloride-containing media, suggesting that chloride waters offer limited protection to nanotoxicity by this mechanism. However, further experiments are required to clarify this, especially as other in vitro studies have shown some protective effect of chloride ions depending on concentration. Our speciation model suggests that soluble dichloride of silver could well act as a source of silver ions, which contribute to toxicity. Oxidative stress is also important and some of this may be directly linked to nanoparticles rather than ionic silver. Overall, this suggests that silver nanoparticles are likely to harm environmental bacteria even at the low concentrations of ionic silver in chloride-containing natural waters.

Speciation in natural waters will be a complex phenomenon as measured/predicted here for chloride-containing waters given the presence of other ligands, redox state and/or residence time of the nanoparticles. In particular, Ag_2S is predicted to form under reducing conditions, while the presence of organic compounds containing thiol functional groups will drive speciation towards formation of stable organic ligands. This can render ionic silver non-bioavailable. Clearly, more work is required to constrain the different environmental variables that influence the bioavailability and toxicity of metal nanoparticles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.12.066.

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