

Differential Gene Regulation in the Ag Nanoparticle and Ag⁺-induced Silver Stress Response in *Escherichia coli*: a Full Transcriptomic Profile

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Abstract

We report the whole-transcriptome response of *Escherichia coli* bacteria to acute treatment with silver nanoparticles (AgNPs) or silver ions (Ag⁺) as silver nitrate using gene expression microarrays. In total, 188 genes were regulated by both silver treatments, 161 were up-regulated and 27 were down-regulated. Significant regulation was observed for heat shock response genes in line with protein denaturation associated with protein structure vulnerability indicating Ag⁺-labile –SH bonds. Disruption to iron-sulfur clusters led to the positive regulation of iron-sulfur assembly systems and the expression of genes for iron and sulphate homeostasis. Further, Ag ions induced a redox stress response associated with large (>600-fold) up-regulation of the *E. coli soxS* transcriptional regulator gene. Ag⁺ is isoelectronic with Cu⁺, and genes associated with copper homeostasis were positively regulated indicating Ag⁺-activation of copper signalling. Differential gene expression was observed for the silver nitrate and AgNP silver delivery. Nanoparticle delivery of Ag⁺ induced the differential regulation of 379 genes; 309 genes were uniquely regulated by silver nanoparticles and 70 genes were uniquely regulated by silver nitrate. The differential silver nanoparticle-silver nitrate response indicates that the toxic effect of labile Ag⁺ in the system depends upon the mechanism of delivery to the target cell.

1 **Introduction**

2 Anti-microbial silver (Ag) is increasingly prevalent in the clinic and in general
3 healthcare (Lansdown, 2006). Specifically, novel silver nanoparticles (AgNPs) are effective
4 broad-spectrum agents that are added to wound dressings, and hygiene products. Their
5 antimicrobial effects are enhanced by a large surface area favouring a high rate of dissolution
6 and release of Ag ions (oxidation of Ag(0) and release of Ag(I)). Dissolved Ag(I) can interact
7 with sulphur- and nitrogen-containing compounds which include protein amino acid side
8 chains (Bauman and Wang, 1964, Vickery and Leavenworth, 1930, Clement and Jarrett,
9 1994, Bell and Kramer, 1999) and metabolically essential iron-sulfur clusters (Fe-S). Thus,
10 the protein targets are potentially pan-metabolic.

11 Bacteria respond to the dissolved Ag(I) by producing small metal-binding proteins
12 that sequester the silver and membrane transporters that remove it from the cytosol. This was
13 first reported for a silver resistant strain of *Salmonella typhimurium* which contained a cluster
14 of plasmid-borne genes encoding dual silver ion exporters and a small soluble silver ion
15 binding protein under the control of a 2-component (Ag(I) sensor- transcriptional responder)
16 signalling system (Gupta, 1999). Orthologues in other species including the
17 enterohaemorrhagic pathogen *Escherichia coli* (Franke, 2001) perform similar roles. The *E.*
18 *coli* *cus* (Cu sensitivity) regulon encodes an RND (Resistance-Nodulation-cell Division)
19 family Ag(I)/Cu(I) exporter (CusCBA) and a small Ag(I)/Cu(I) binding protein (CusF)
20 (Kittleson et al., 2006, Franke et al., 2003). The genes are over-expressed in silver resistant
21 strains (Lok et al., 2008a) and inactivation in the wild-type is consistent with increased
22 sensitivity (Franke, 2001). The association with copper is logical as the Ag(I) and Cu(I) ions
23 have the same d^{10} electron configuration, similar charge and ionic radii. However, there is no
24 evidence to suggest that a second copper export system in *E. coli*, CopA, has any effect on
25 silver tolerance. Silver resistant strains of *E. coli* raised in the laboratory lack a sub-set of
26 constitutive outer membrane Porin proteins, OmpC and OmpF, indicating a chemiosmotic
27 defence, but gene knockout mutants had no detectable sensitivity compared to the wild type
28 (Li et al., 1997).

29 Previous studies have addressed the role of a limited sub-set of *E. coli* genes in
30 response to Ag(I) in solution but the potentially pan-metabolism action of Ag(I) on proteins
31 alludes to large-scale genetic regulation. For AgNPs, the toxic mechanism may be enhanced
32 by association of the nanoparticle and bacterial surface and the subsequent localised
33 dissolution and ion release directly against the cell wall. In our earlier study, we reported that
34 the AgNP toxicity mechanism induces a quantitatively greater transcriptional response to

1 silver stress than Ag(I) added as silver nitrate, even though the measured bulk solution phase
2 Ag(I) concentration was the same. This study was restricted to a sub-set of *E. coli* Ag-
3 responding genes but differences in the global genetic response were not investigated
4 (McQuillan et al., 2012). In eukaryotes, including *Saccharomyces cerevisiae*, a differential
5 dissolved Ag(I)-AgNP response has been measured using microarrays (Niazi et al., 2011,
6 Kawata et al., 2009, Roh et al., 2009, Lim et al., 2012) but to our knowledge, these
7 experiments have never been performed in prokaryotes, which are clearly an important target
8 group. In this study, we report the findings from whole transcriptome gene expression
9 microarray experiments to capture the overall genetic response to (a) 142 nm AgNPs and (b)
10 AgNO₃ in the Gram negative bacterium, *E. coli* K12. The response was measured at the early
11 stage 10 minutes following silver shock at a sub-inhibitory dose to reduce background gene
12 regulation from secondary effects including a change in growth phase. Genes that responded
13 to both treatments are described in terms of the response to dissolved Ag(I), the common
14 toxicant, and we report on genes that responded differentially in the two treatments reflecting
15 differences in the mechanism of action for the two physical forms of Ag.

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1 **Methods**

2 *Materials*

3 All reagents were purchased from Sigma-Aldrich unless otherwise stated. The Silver
4 Nanoparticles (AgNPs) were synthesised in the vapour phase (QinetiQ Nanomaterials Ltd,
5 Farnborough, UK) and supplied as a dry powder. The mean equivalent spherical diameter
6 was 142 ± 20 nm (mean \pm standard error of the mean), determined in transmission electron
7 images after dispersion in the experimental medium (low-salt Luria broth as defined below)
8 using a JEOL 1400 TEM. The specific surface area was determined by BET adsorption
9 isotherm and was $4 \text{ m}^2/\text{g}$. Scanning Electron Microscopy with Energy dispersive X-ray
10 (EDX) analysis was carried out to confirm that the nanoparticles were silver with no other
11 elements detected (HITACHI S3200N SEM fitted with EDAX detection; INCA, Oxford
12 Instruments). The characterisation data including an assessment of the antibacterial activity of
13 this specific material batch has been determined previously (McQuillan et al., 2012).

14

15 *Bacterial Culture and Ag Treatment*

16 *E. coli* K12 (MG1655) was received from the Coli Genetic Stock Centre and
17 maintained on Luria agar at 37°C. All cultures were carried out in a low-salt Luria broth (LB)
18 which was 10 g Tryptone and 5 g yeast extract in 1 L of water and pH 7.5. The salt was
19 omitted as this improved the colloidal stability of the AgNPs and reduced precipitation of
20 AgCl, but the medium still supported rapid growth and replication of the *E. coli*. Nanoparticle
21 dispersion in the LB was achieved by sonicating the mixture for 2 minutes using a Soniprep
22 150 (MSE Instruments, London, UK). The bacterial growth curve was determined for a 100
23 mL culture, under aerobic conditions at 37°C with constant agitation. Viable cell numbers
24 were measured at 30 minute intervals using the plate counting method. For AgNP treatment
25 the dry nanopowder was dispersed in 10 mL of the LB by sonication at 10 \times the required
26 concentration, then diluted to 100 mL with a log-phase culture of the *E. coli* containing 10^7
27 cfu/mL. For silver nitrate we used an identical procedure; log-phase cultures were diluted
28 with fresh medium containing a 10 \times concentrated solution of AgNO₃. The exposure
29 concentration was 40 $\mu\text{g}/\text{mL}$ for AgNPs or 0.4 $\mu\text{g}/\text{mL}$ for Ag(I) as AgNO₃. Control cultures
30 were similarly diluted at the time of exposure and each Ag treatment was performed using
31 quadruplicate treated/untreated control pairs.

32

33 *Microarray Experiments*

1 RNA was stabilised and isolated from each culture using the RNAsprotect Reagent
2 with the RNeasy Mini Kit (Qiagen, Crawley, UK). Residual DNA was digested with RQ1
3 RNase free DNase (Promega, Southampton, UK). The complete removal of the DNA was
4 confirmed by a null result in a Taq polymerase-based PCR from the samples, wherein Taq is
5 a DNA-specific polymerase and cannot amplify from an RNA template.. The RNA was
6 purified using the RNeasy clean-up protocol and analysed by agarose electrophoresis and
7 spectrophotometry. High quality RNA was amplified, reverse transcribed and labelled (Cy3
8 for treated and Cy5 for untreated, including at least one dye swap) using the MessageAmp-II
9 Bacteria Kit (Applied Biosystems, Warrington, UK). Hybridisation was carried out according
10 to the instructions of the microarray manufacturer (Agilent Technologies, USA). Briefly, the
11 labelled probes were mixed with fragmentation and blocking buffer at 60°C for 30 minutes.
12 The fragmentation reaction was terminated by mixing (1:1) with a hybridisation buffer
13 containing 25% formamide, 5× Saline Sodium Citrate, 0.1% Sodium Dodecyl Sulfate and 1
14 % salmon sperm DNA. Then, 40 µL of hybridization sample was loaded onto each array
15 using the SureHyb assembly apparatus. For each Ag treatment, the quadruplicate
16 treated/untreated control pairs were hybridised with quadruplicate gene expression
17 microarrays (Product G4813A-020097) which were printed on glass in an 8 by 15,000 feature
18 format. The hybridization reaction was carried out in a rotisserie oven at 65°C for 17 hours.
19 The array was washed with Agilent gene expression wash buffers in a 1 L staining dish that
20 had been cleaned with acetonitrile and ultrapure water. All steps were carried out in an ozone
21 controlled environment.

22

23 *Data Analysis*

24 The microarray slide was scanned on a GenePix 4000B array scanner (Molecular
25 Devices, USA) and feature extraction was carried out with Agilent Feature Extraction
26 software (Agilent Technologies, USA). Defective spots were excluded and the dye intensity
27 for each spot was normalised using local background subtraction. Overall normalisation of
28 dye intensity bias was performed using the global ‘within array’ LOWESS method. Gene
29 expression filtering and statistical analysis was carried out using GeneSpring (Agilent
30 Technologies, USA). Genes were filtered by excluding those whose expression failed to
31 change by more than 2-fold. The remaining genes were subject to confidence testing using
32 the t-test with correction for multiple testing using the Benjamini-Hochberg False Discovery
33 Rate (Benjamini and Hochberg, 1995). Gene Ontology clustering and enrichment analysis
34 was performed using GeneCoDis (Carmona-Saez et al., 2007, Nogales-Cadenas et al., 2009).

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Real Time PCR

The microarray data were validated by real-time PCR. First strand cDNA was synthesised using the same RNA samples and the Thermoscript RT System (Invitrogen, Paisley, UK) Real time PCR was performed on a select gene set using the Stratagene MxPro system and the SYBR green DNA detection chemistry (Biorad, Hemel Hempstead, UK). All RT-PCR experiments were repeated in triplicate. Data were analysed according to the method of Pfaffl (Pfaffl, 2001), using a dilution series based on pooled cDNA samples to determine the primer efficiency. The internal reference gene was *rrsB*, encoding the 16s rRNA subunit, which is not regulated by Ag. The primer pairs were as follows. For *cueO*: Forward, TACCGATCCCTGATTTGCTC, Reverse, GACTTCACCCGGTACTTCCA; *cusA*: Forward, TGGATGGGCTTTCATCTTTC, Reverse, TTCTGCTCGCTGAATGTTTG; *ompF*: Forward, TGCGCAACTAACAGAACGTC, Reverse, AGGCTTTGGTATCGTTGGTG; *soxS*: Forward, GTAATCGCCAAGCGTCTGAT, Reverse, CCCATCAGAAAATTATTCAGGATCT. Primers were designed to amplify a 200-300 bp region of the target gene.

1 **Results**

2 The microarrays measured the global changes in gene expression in exponentially
3 replicating (log-phase) *E. coli* after 10 minutes exposure to 142 nm AgNPs or AgNO₃. The
4 nanoparticles have been characterised in our earlier study (McQuillan et al., 2012) and are
5 composed of silver, with no surface ligand. They dissolve in the experimental medium at a
6 rate that is linearly related to the surface area, associate directly with the cell surface and are
7 acutely toxic to the *E. coli*. The bacteria were treated with 40 µg/mL of AgNPs or 0.4 µg.mL
8 of Ag⁺ for precisely 10 minutes in a rich medium (low salt Luria Broth). The dose did not
9 inhibit bacterial replication in order to avoid background gene regulation associated with a
10 change in growth phase, Figure 1.

11 After the 10 minute exposure the mRNA pool was stabilised and used to synthesise
12 cDNA labelled with Cy3 (control samples) or Cy5 (Ag treated samples) including at least one
13 dye swap per experiment. Treated/untreated control pairs were hybridised with Agilent gene
14 expression microarrays. Gene regulation was subject to confidence testing and filtered using
15 a >2-fold change cut-off to generate lists of significantly up-regulated and down-regulated
16 genes, summarised in Table 1. In total, 188 genes were regulated in both Ag treatments, 161
17 were up-regulated and 27 were down-regulated. However, the response to each treatment was
18 also clearly different; 309 genes were regulated exclusively by AgNPs whereas only 70 genes
19 were regulated exclusively by silver nitrate and overall the response to AgNPs was almost 2-
20 fold greater in magnitude.

21 Biological interpretation of the microarray data was carried out using GeneCoDis to
22 find significantly enriched (hypergeometric test, $p \leq 0.05$) Gene Ontology (GO) terms against
23 a whole-genome reference set containing 4,619 *E. coli* genes. GO term enrichment analysis
24 was carried out for the lists of up- and down-regulated genes, regulated by both treatments or
25 regulated independently. The results of the enrichment analysis are summarised in Figure 2.
26 The full gene lists are given in the supplementary information, Table S1 (AgNPs) and Table
27 S2 (silver nitrate). Genes which we refer to in our discussion of the results are presented in
28 Tables 2-6.

29 The microarray data were validated by comparing the expression ratio of 4 genes
30 (*ompF*, *cueO*, *cusF* and *soxS*) with the results of expression analysis using real-time PCR for
31 the same RNA samples. We found that the results from microarray analysis and real-time
32 PCR had a strong correlation, Figure 3.

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1 Discussion

2 The overall genetic response to the two physical forms of silver was quite different. In
3 total 379 genes were differentially regulated; 309 genes were only regulated by AgNPs and
4 70 genes were only regulated by silver nitrate. For both forms of Ag, the primary toxicant is
5 Ag(I). For Ag added as AgNO₃ the labile Ag(I) can form Ag-complexes with components in
6 the medium and labile Ag ions enter the *E. coli* through the cation selective outer membrane
7 porin proteins. Dissolved Ag(I) is also supplied to the medium from disperse AgNPs which
8 dissolve following oxidation of the silver surfaces. Therefore, we expect that the differential
9 response is a result of additive toxic effects from the Ag(I) delivery mechanism. In
10 Eukaryotes, an AgNP toxicity process independent of ion release is described for
11 imperfections in the crystal lattice structure and highly reactive electron configurations at the
12 NP surface (George et al., 2012, Nel et al., 2006). However, there is no evidence to support
13 this in bacteria, which have structurally and chemically distinct membranes, in line with
14 recent evidence that AgNPs have no anti-bacterial activity if ion release is blocked under
15 anaerobic conditions (Xiu et al., 2012). In our earlier study, we proposed a hypothesis based
16 on the observation that the nanoparticles associate directly with the cell surface, and dissolve
17 on the outer membrane to create a high interfacial Ag(I) concentration, which enhances the
18 anti-bacterial effects as a function of the labile Ag(I) concentration in the bulk solution of the
19 medium (McQuillan et al., 2012). Accordingly in our Ag exposures, the AgNP-treated
20 bacteria may experience membrane proximity damage and a gradient of Ag(I) from the
21 dissolving nanoparticle acting as a point source. In contrast, exposure to silver nitrate may
22 generate entirely different concentration gradients of Ag(I)⁺ within the cell. AgNPs with a
23 diameter of 12 nm, far smaller than those used in this study, have been shown to penetrate
24 into the cell wall and enter the cytoplasm of *E. coli*, and interact directly with nucleic acids
25 (Sondi and Salopek-Sondi, 2004, Jose Ruben and et al., 2005) which could further
26 differentiate the AgNP-AgNO₃ toxicity. However, we consider this process unlikely to occur
27 under these conditions because (1) the nanoparticles are relatively large (142 nm) and (2)
28 sufficient membrane damage to allow entry of a particle this size would be lethal to the cell
29 whereas the dose used in our experiments was sub-inhibitory. There are no known
30 nanoparticle transport processes in *E. coli* or other prokaryotes that could facilitate uptake in
31 this size range.

32 The biological interpretation of the differentially regulated genes gave no clear
33 indication as to why the responses were different. Genes for lipid and fatty acid biosynthesis
34 were down-regulated only after exposure to AgNPs but we would predict that localised

1 membrane damage would lead to an increase in the expression of these genes. If the cell
2 cycle is temporarily arrested upon sudden addition of Ag, the lipid biosynthetic processes
3 may be reduced and captured in the early phase 10 minute response. Although the overall
4 growth profiles after exposure to both forms of Ag are equivalent, Ag(I) added as AgNO₃
5 has the greater lability in the medium and the initial metabolism response to AgNPs may
6 have been delayed.

7 In total 188 genes were regulated after exposure to both forms of Ag; 161 genes were
8 up-regulated and 27 genes were down-regulated. This response follows a logical pattern for
9 the indiscriminate action of Ag(I) on proteins, leading to potentially pan-metabolism toxic
10 effects which require a substantial regulation of the *E. coli* genome. This was up to 11.1 % of
11 the identified genes after applying a 2-fold change cut-off and confidence filters. Studies on
12 silver sulfadiazine, a topical agent for anti-sepsis of superficial burns, demonstrate that Ag(I)
13 can also bind to nucleic acids (Rosenkranz and Carr, 1972, Rosenkranz and Rosenkranz,
14 1972) but DNA is typically localised to the core of the cell and surrounded by high
15 concentrations of proteins which will be attacked first. Accordingly, at the sub-inhibitory
16 dose used in our microarray experiments there was no evidence for a genotoxic response.

17 The action of Ag(I) on protein structure led to the induction of the *E. coli* heat-shock
18 response (HSR) and the positive regulation of genes encoding protein chaperones and
19 proteolytic enzymes for the stabilisation and re-folding or proteolysis of denatured
20 polypeptides. Protein molecular chaperones DnaK-DnaJ-GrpE (DJE) and GroEL-GroES
21 were induced by up to 19-fold, and genes encoding the small heat shock proteins IbpA and
22 IbpB by up to 180-fold, Table 2. At the same 10 minute time point there was down-regulation
23 of genes associated with translation, consistent with the requirement to mount an adaptive
24 response before consuming cellular resources to generate more proteins (Lindquist, 1981).
25 The HSR is positively regulated by a sudden increase in the cytosolic concentration of the
26 sigma 32 (σ^{32}) subunit of RNA polymerase (RNAP). Regulation of the response by Ag could
27 be based on the competitive binding of Ag-denatured protein substrates and σ^{32} with the
28 protein chaperone DnaK (Arsene et al., 2000, Bukau, 1993), which allows for a temperature
29 independent activation of the HSR genes.

30 Ag also induced genes belonging to the operons *isc* and *suf*, encoding iron-sulfur
31 cluster assembly proteins (Py and Barras, 2010), Table 3. This demonstrates that Ag⁺ can
32 perturb Fe-S metabolism in line with evidence that Ag causes uncoupling of the respiratory
33 chain and respiratory arrest (Holt and Bard, 2005). Predictably, the biosynthesis of new Fe-S

1 clusters increases the cellular demand for iron and the positive regulation of genes under the
2 control of the Ferric Uptake Regulator (Fur) regulon which increase the supply of ferric iron
3 (Fe^{3+}) from the medium, Table 4. The low iron response is enhanced by the expression of
4 *cueO*, which was up-regulated by up to 320-fold. CueO is a Cu(I)/Ag(I)-inducible cuprous
5 oxidase which can oxidise and inactivate the enterobactin siderophore (Grass et al., 2004),
6 reducing the rate of iron acquisition. Accordingly, the same low iron response is stimulated
7 by excess copper (Kershaw et al., 2005). Ag also induced the genes *cysA* and *cysW*, which
8 encode subunits of the ABC family sulphate/thiosulfate transporter, and a complement of
9 genes required for intracellular sulphate reduction and assimilation during the biosynthesis of
10 cysteine, Table 5. Activation of cysteine biosynthesis is logical as the functional thiol side
11 chain is a specific molecular target for Ag(I), and the supply of sulfur would further support
12 the assembly of novel Fe-S.

13 As the Fur regulator protein responds directly to cytosolic Fe(II) concentration the
14 activation of this regulon indicates that the cytosolic pool of iron is depleted quickly, within
15 the 10 minutes following Ag exposure. For pathogenic *E. coli* the availability of iron is a key
16 factor in virulence associated with successful colonisation of the urinary tract and
17 proliferation in the small intestine (Litwin and Calderwood, 1993). Destruction of essential
18 iron-sulfur proteins and an increase in the iron requirement could represent a fundamental
19 anti-bacterial mechanism for Ag *in vivo* where iron availability is necessarily kept minimal as
20 part of the innate host defences.

21 Another mechanism in Ag toxicity might be to displace metabolically important
22 metal ion cofactors from their native coordination sites on proteins. Specifically the parallels
23 between silver and copper chemistry in *E. coli* are well established (Franke et al., 2001,
24 Franke et al., 2003, Loftin et al., 2007). The Ag(I) and Cu(I) ions have the same d^{10} electronic
25 configuration, charge and similar ionic radii, and have been shown to have a similar protein
26 coordination chemistry (Loftin et al., 2007). If Ag(I) displaces Cu(I) from its native
27 coordination sites on proteins then the labile Cu(I) released into the cell may lead to the
28 generation of hydroxyl radicals (Simpson et al., 1988). Our microarray results indicate that
29 Ag(I) may interact with Cu(I) sensor proteins, CusS and CueR, which activate genes
30 encoding copper ion homeostasis systems; CusCFBA, CopA and CueO (Franke et al., 2003,
31 Lok et al., 2008b, Munson et al., 2000). Both forms of Ag induced a complement of CusS
32 and CueR regulated genes, Table 6, but interestingly, the metal ion binding domain of CusS
33 is located on the periplasmic face of the plasma membrane whereas CueR is a soluble

1 cytosolic protein, so activation by Ag treatment indicates that dissolved Ag(I) could have
2 been present in multiple cellular compartments.

3 Labile copper displaced from cupro-protein complexes could undergo redox cycling
4 to generate highly reactive oxygen radicals, in line with evidence that AgNPs induce
5 oxidative stress responses in human hepatoma cells (Kim et al., 2009), zebra fish hepatocytes
6 (Choi et al.), fruit fly larvae (Ahamed et al., 2009) and in the bacterium *Staphylococcus*
7 *aureus* (Dagmar Chudobova, 2013). In the *E. coli* model, a small complement of antioxidant
8 systems belonging to the *soxRS* regulon were expressed at high levels but there was no
9 significant enrichment of redox stress-associated GO annotations in the gene lists. The SoxR
10 protein is a constitutive cytosolic transcription factor which is activated following oxidation
11 of iron-sulfur clusters [2Fe-2S], and could be directly compromised by Ag(I). Active SoxR
12 positively regulates the expression of *soxS*, expressed by up to 600-fold following Ag
13 treatment, encoding a second transcription factor that acts sequentially to initiate a cascade of
14 anti-oxidant responses. The high level of gene induction for *soxS*, which was up-regulated
15 more than any other gene, is evidence that redox stress is an important determinant of Ag
16 toxicity.

17

18 **Conclusions**

19 In conclusion, our data for the differential AgNP-AgNO₃ response support a growing
20 body of evidence for a nanoparticle-specific silver ion dependent toxicity mechanism. We
21 propose, based upon our earlier observations of AgNPs dissolving in the medium and
22 attaching to the cell surface (McQuillan et al., 2012), that dissolution at the cell wall produces
23 an enhanced interfacial concentration that enters the cell. We have previously shown that
24 nanoparticle-delivery can enhance the anti-bacterial activity of Ag(I), but this is only
25 applicable if AgNPs are free to interact with the cell surface. Products which contain AgNPs
26 fixed in a gel matrix (Jain et al., 2009) that cannot interact directly with bacteria may not
27 benefit from this enhanced effect. For the overall genetic response to both physical forms of
28 Ag the comprehensive induction of genes for the heat shock response is evidence that Ag(I)
29 acts on protein structure, and consistent with genetic responses to silver in eukaryotic models
30 *Drosophila melanogaster* (Ahamed et al., 2009), and *Caenorhabditis elegans* (Roh et al.,
31 2009). The unfolded protein response has been linked with serious disease in humans and
32 prolonged non-essential silver use should be monitored. Additional disruption occurs at iron-
33 sulfur components leading to disruption of metabolically essential processes and could
34 represent the critical Ag target, leading to respiratory arrest and a demand for iron, which is

1 typically a limiting nutrient in various *E. coli* infection scenarios. There was clear evidence
2 that Ag causes redox stress but the greatest response in terms of the number of genes
3 regulated was the response to unfolded proteins, reflecting the pan-metabolism action of
4 Ag(I) on protein structure and function. Accordingly, we consider that this process is the
5 primary mechanism in Ag toxicity against *E. coli*.

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3

1 **Figure Legends**

2 **Figure 1.** Growth Plots for the *E. coli* in low salt Luria Broth. The solid line shows the
3 growth plot for the *E. coli* in the low salt Luria Both. The bacteria were treated with Ag when
4 the cultures reached a density of approximately 10^7 CFU/mL (2 hours). The dashed lines
5 show the sub-inhibitory effect of the exposure concentration for AgNPs (\square) and Ag(I) as
6 AgNO₃ (Δ) on the post-exposure growth plot. The error bars represent the standard error of
7 the mean for triplicate cultures.

8

9 **Figure 2.** GO Term Enrichment Clustering Analysis using GeneCoDis. Bar charts showing
10 the number of genes sharing specific Gene Ontology (GO) terms that were significantly
11 enriched in the lists of up-regulated (top) or down regulated (bottom) genes including those
12 that were independently regulated by AgNPs or Ag(I) as AgNO₃. The bar labels include the
13 term accession number.

14

15 **Figure 3.** Validation of microarray data by real-time PCR. The microarray gene expression
16 data were validated by comparing the results with a set of genes (*ompF*, *cusA*, *cueO* and
17 *soxS*) which were measured by real-time PCR using the same RNA samples. In both
18 experiments, either (A) Ag(I) as AgNO₃ or (B) AgNPs linear regression analysis shows that
19 the expression values had a strong correlation with R² values of 0.987 and 0.983 respectively.
20 The units are Log 2 gene expression ratio between Ag treated and untreated control samples.

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1 **Tables**

Table 1. Gene Regulation in *E. coli* Exposed to AgNO₃ or AgNPs at 10 minutes.

	Total genes regulated >2-fold				Genes uniquely regulated >2-fold			
	Up Regulated	Down Regulated	Total	% of Genome	Up regulated	Down Regulated	Total	% of Genome
Silver Nanoparticles	273	224	497	11.1 %	112	197	309	6.9 %
Silver Nitrate	220	38	258	5.8 %	59	11	70	1.7 %

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Table 2. Heat Shock Response Genes Induced by Silver

Gene	Function	Expression Ratio	
		AgNO ₃	AgNPs
<i>ipaA</i>	Small heat shock proteins; bind and stabilise denatured polypeptides	180.16	6.44
<i>ipaB</i>		4.76	17.55
<i>clpB</i>	Disaggregation of insoluble protein aggregates	83.16	12.66
<i>clpP</i>	Proteolysis	2.04	3.23
<i>dnaK</i>	DnaK-DnaJ-GrpE (DJE) complex; chaperone for protein folding; protein disaggregation; regulation of the heat shock response	19.32	6.62
<i>dnaJ</i>		3.46	2.06
<i>grpE</i>		4.96	4.25
<i>groS</i>	GroEL complex; chaperone for protein folding; protein re-folding	9.93	5.69
<i>groL</i>		9.44	5.44
<i>htpG</i>	Protein folding; homologue to mammalian HSP90	11.33	5.97
<i>htpX</i>	Protease; degradation of denatured polypeptides	8.94	5.47
<i>hslJ</i>	Heat shock locus proteins	*	5.91
<i>hslO</i>		3.62	3.37
<i>hslR</i>		2.90	2.62
<i>hslU</i>		2.36	2.28
<i>hslV</i>		2.33	2.72
<i>lon</i>		DNA-binding ATP-dependent protease	2.85
<i>idhA</i>	NAD-linked fermentative lactate dehydrogenase; associated with heat stress	3.01	2.48
<i>hflK</i>	Putative proteases; associated with heat stress	2.88	2.31
<i>hflX</i>		*	2.41
<i>rrmJ</i>	Ribosome associated methyltransferase; associated with heat stress	2.76	3.12
<i>cpxR</i>	CpxAR regulon; responds to protein unfolding in the periplasm; responds to Cu ⁺	*	2.80
<i>ppiA</i>		*	2.67
<i>dsbA</i>		*	2.57

1 * failed expression cut-off or confidence filters

2

Table 3. Iron-Sulfur Cluster Assembly Genes Induced by Silver

Gene	Function	Expression Ratio	
		AgNO₃	AgNPs
<i>iscA</i>		4.11	2.09
<i>iscU</i>	Fe-S assembly complex	3.65	2.06
<i>iscS</i>		3.14	*
<i>iscR</i>	Regulatory protein for <i>iscSUA</i>	7.99	2.68
<i>sufA</i>		62.29	16.26
<i>sufB</i>		30.58	10.82
<i>sufC</i>	Fe-S assembly complex	20.58	11.97
<i>sufD</i>		18.06	7.83

1 * failed expression cut-off or confidence filters

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Table 4. Fur Regulon Genes Induced by Silver

Gene	Function	Expression Ratio	
		AgNO ₃	AgNPs
<i>entA</i>		146.78	9.19
<i>entB</i>		116.39	10.65
<i>entC</i>		161.96	4.91
<i>entD</i>	Biosynthesis of the enterobactin siderophore	6.82	4.69
<i>entE</i>		77.22	12.95
<i>entF</i>		53.56	15.07
<i>ybdB</i>		160.24	7.49
<i>Cir</i>	Outer Membrane Fe ³⁺ /ligand receptors	386.65	4.44
<i>Fiu</i>		41.44	3.32
<i>fepA</i>		47.48	12.21
<i>fepB</i>		55.40	2.87
<i>fepC</i>	Uptake of Fe ³⁺ /enterobactin	24.10	*
<i>fepD</i>		20.76	*
<i>fepG</i>		27.12	*
<i>fecR</i>		29.91	2.05
<i>fecI</i>	Uptake of ferric citrate	35.64	2.27
<i>fecA</i>		5.21	*
<i>fecB</i>		2.99	*
<i>fhuA</i>		14.38	*
<i>fhuC</i>	Uptake of ferrichrome	5.62	*
<i>fhuE</i>		20.93	16.07
<i>fhuF</i>		19.91	*
<i>tonB</i>		2.43	*
<i>exbB</i>	Inner membrane complex; energises the outer membrane Fe ³⁺ /ligand transporters	4.19	*
<i>exbD</i>		4.03	*

1 * failed expression cut-off or confidence filters

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Table 5. Sulfate Transport and Assimilation Genes Induced by Silver

Gene	Function	Expression Ratio	
		AgNO₃	AgNPs
<i>cysA</i>		16.19	6.50
<i>cysC</i>		4.29	5.94
<i>cysD</i>		4.49	7.00
<i>cysH</i>		13.66	6.97
<i>cysI</i>	Sulfate assimilation and biosynthesis of cysteine	8.49	7.83
<i>cysK</i>		3.70	2.49
<i>cysN</i>		23.75	6.74
<i>cysP</i>		2.02	2.72
<i>cysW</i>		5.54	5.22

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Table 6. Copper Homoestasis Genes Induced by Silver

Gene	Function	Expression Ratio	
		AgNO ₃	AgNPs
<i>copA</i>	P-type ATPase; copper transporter	47.09	13.71
<i>cueO</i>	Periplasmic cuprous oxidase	320.45	36.72
<i>cusC</i>		72.48	40.54
<i>cusF</i>	RND-protein driven cation/proton exchanger; may transport Cu ⁺ and Ag ⁺ ; <i>cusF</i>	376.39	54.08
<i>cusB</i>	encodes a small Ag ⁺ /Cu ⁺ binding protein	99.98	26.90
<i>cusA</i>		50.32	20.72
<i>cusR</i>	Two-component regulatory system; regulates <i>cusCFBA</i>	2.53	5.11
<i>cusS</i>		*	2.83

1 * failed expression cut-off or confidence filters

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1 References

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- 3 AHAMED, M., POSGAI, R., GOREY, T. J., NIELSEN, M., HUSSAIN, S. M. & ROWE, J. J. 2009. Silver
4 nanoparticles induced heat shock protein 70, oxidative stress and apoptosis in *Drosophila*
5 *melanogaster*. *Toxicology and Applied Pharmacology*, 242, 263-269.
- 6 ARSENE, F., TOMOYASU, T. & BUKAU, B. 2000. The heat shock response of *Escherichia coli*. *Int J Food*
7 *Microbiol*, 55, 3-9.
- 8 BAUMAN, J. E. & WANG, J. C. 1964. Imidazole Complexes of Nickel(II), Copper(II), Zinc(II), and
9 Silver(I). *Inorganic Chemistry*, 3, 368-373.
- 10 BELL, R. A. & KRAMER, J. R. 1999. Structural chemistry and geochemistry of silver-sulfur compounds:
11 Critical review. *Environmental Toxicology and Chemistry*, 18, 9-22.
- 12 BENJAMINI, Y. & HOCHBERG, Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful
13 Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B*
14 *(Methodological)*, 57, 289-300.
- 15 BUKAU, B. 1993. Regulation of the *Escherichia coli* heat-shock response. *Molecular Microbiology*, 9,
16 671-680.
- 17 CARMONA-SAEZ, P., CHAGOYEN, M., TIRADO, F., CARAZO, J. M. & PASCUAL-MONTANO, A. 2007.
18 GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists.
19 *Genome Biol*, 8, R3.
- 20 CHOI, J. E., KIM, S., AHN, J. H., YOUN, P., KANG, J. S., PARK, K., YI, J. & RYU, D.-Y. Induction of
21 oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. *Aquatic*
22 *Toxicology*, 100, 151-159.
- 23 CLEMENT, J. L. & JARRETT, P. S. 1994. Antibacterial silver. *Met Based Drugs*, 1, 467-82.
- 24 DAGMAR CHUDOBOVA, J. D., LUKAS NEJDL, DARINA MASKOVA, MIGUEL ANGEL MERLOS RODRIGO,
25 BRANISLAV-RUTTKAY NEDECKY, OLGA KRYSOFOVA, JINDRICH KYNICKY, MARIE KONECNA,
26 MIROSLAV POHANKA, JAROMIR HUBALEK, JOSEF ZEHNALIK, BORIVOJ KLEJDUS, RENE KIZEK,
27 VOJTECH ADAM 2013. Oxidative Stress in *Staphylococcus aureus* Treated with Silver(I) Ions
28 Revealed by Spectrometric and Voltammetric Assays. *International Journal of*
29 *Electrochemical Science*, 8.
- 30 FRANKE, S., GRASS, G. & NIES, D. H. 2001. The product of the ybdE gene of the *Escherichia coli*
31 chromosome is involved in detoxification of silver ions. *Microbiology*, 147, 965-72.
- 32 FRANKE, S., GRASS, G., RENSING, C. & NIES, D. H. 2003. Molecular analysis of the copper-
33 transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol*, 185, 3804-12.
- 34 FRANKE, S., GRASS, G. NIES, D. H. 2001. The Product of the ybdE Gene of the *Escherichia coli*
35 Chromosome is Involved in the Detoxification of Silver Ions. *Microbiology*, 147, 965-972.
- 36 GEORGE, S., LIN, S., JI, Z., THOMAS, C. R., LI, L., MECKLENBURG, M., MENG, H., WANG, X., ZHANG, H.,
37 XIA, T., HOHMAN, J. N., LIN, S., ZINK, J. I., WEISS, P. S. & NEL, A. E. 2012. Surface defects on
38 plate-shaped silver nanoparticles contribute to its hazard potential in a fish gill cell line and
39 zebrafish embryos. *ACS Nano*, 6, 3745-59.
- 40 GRASS, G., THAKALI, K., KLEBBA, P. E., THIEME, D., MULLER, A., WILDNER, G. F. & RENSING, C. 2004.
41 Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia*
42 *coli*. *J Bacteriol*, 186, 5826-33.
- 43 GUPTA, A., MATSUI, K., LO, J. F., SILVER, S. 1999. Molecular Basis for Resistance to Silver Cations in
44 *Salmonella*. *Nature Medicine*, 5, 183-188.
- 45 HOLT, K. B. & BARD, A. J. 2005. Interaction of silver(I) ions with the respiratory chain of *Escherichia*
46 *coli*: an electrochemical and scanning electrochemical microscopy study of the antimicrobial
47 mechanism of micromolar Ag⁺. *Biochemistry*, 44, 13214-23.
- 48 JAIN, J., ARORA, S., RAJWADE, J. M., OMRAY, P., KHANDELWAL, S. & PAKNIKAR, K. M. 2009. Silver
49 nanoparticles in therapeutics: development of an antimicrobial gel formulation for topical
50 use. *Mol Pharm*, 6, 1388-401.

- 1 JOSE RUBEN, M. & ET AL. 2005. The bactericidal effect of silver nanoparticles. *Nanotechnology*, 16,
2 2346.
- 3 KAWATA, K., OSAWA, M. & OKABE, S. 2009. In Vitro Toxicity of Silver Nanopartides at Noncytotoxic
4 Doses to HepG2 Human Hepatoma Cells. *Environmental Science & Technology*, 43, 6046-
5 6051.
- 6 KERSHAW, C. J., BROWN, N. L., CONSTANTINIDOU, C., PATEL, M. D. & HOBMAN, J. L. 2005. The
7 expression profile of Escherichia coli K-12 in response to minimal, optimal and excess copper
8 concentrations. *Microbiology*, 151, 1187-1198.
- 9 KIM, S., CHOI, J. E., CHOI, J., CHUNG, K.-H., PARK, K., YI, J. & RYU, D.-Y. 2009. Oxidative stress-
10 dependent toxicity of silver nanoparticles in human hepatoma cells. *Toxicology in Vitro*, 23,
11 1076-1084.
- 12 KITTLESON, J. T., LOFTIN, I. R., HAUSRATH, A. C., ENGELHARDT, K. P., RENSING, C. & MCEVOY, M. M.
13 2006. Periplasmic metal-resistance protein CusF exhibits high affinity and specificity for both
14 Cul and AgI. *Biochemistry*, 45, 11096-102.
- 15 LANSDOWN, A. B. 2006. Silver in health care: antimicrobial effects and safety in use. *Curr Probl
16 Dermatol*, 33, 17-34.
- 17 LI, X. Z., NIKAIDO, H. & WILLIAMS, K. E. 1997. Silver-resistant mutants of Escherichia coli display
18 active efflux of Ag⁺ and are deficient in porins. *J Bacteriol*, 179, 6127-32.
- 19 LIM, D.-H., JANG, J., KIM, S., KANG, T., LEE, K. & CHOI, I.-H. 2012. The effects of sub-lethal
20 concentrations of silver nanoparticles on inflammatory and stress genes in human
21 macrophages using cDNA microarray analysis. *Biomaterials*, 33, 4690-4699.
- 22 LINDQUIST, S. 1981. Regulation of protein synthesis during heat shock. *Nature*, 293, 311-314.
- 23 LITWIN, C. M. & CALDERWOOD, S. B. 1993. Role of iron in regulation of virulence genes. *Clinical
24 Microbiology Reviews*, 6, 137-149.
- 25 LOFTIN, I. R., FRANKE, S., BLACKBURN, N. J. & MCEVOY, M. M. 2007. Unusual Cu(I)/Ag(I) coordination
26 of Escherichia coli CusF as revealed by atomic resolution crystallography and X-ray
27 absorption spectroscopy. *Protein Sci*, 16, 2287-93.
- 28 LOK, C.-N., HO, C.-M., CHEN, R., TAM, P. K.-H., CHIU, J.-F. & CHE, C.-M. 2008a. Proteomic
29 Identification of the Cus System as a Major Determinant of Constitutive Escherichia coli
30 Silver Resistance of Chromosomal Origin. *Journal of Proteome Research*, 7, 2351-2356.
- 31 LOK, C. N., HO, C. M., CHEN, R., TAM, P. K., CHIU, J. F. & CHE, C. M. 2008b. Proteomic identification
32 of the Cus system as a major determinant of constitutive Escherichia coli silver resistance of
33 chromosomal origin. *J Proteome Res*, 7, 2351-6.
- 34 MCQUILLAN, J. S., INFANTE, H. G., STOKES, E. & SHAW, A. M. 2012. Silver nanoparticle enhanced
35 silver ion stress response in Escherichia coli K12. *Nanotoxicology*, 6, 857-66.
- 36 MUNSON, G. P., LAM, D. L., OUTTEN, F. W. & O'HALLORAN, T. V. 2000. Identification of a Copper-
37 Responsive Two-Component System on the Chromosome of Escherichia coli K-12. *J.
38 Bacteriol.*, 182, 5864-5871.
- 39 NEL, A., XIA, T., MÄDLER, L. & LI, N. 2006. Toxic Potential of Materials at the Nanolevel. *Science*, 311,
40 622-627.
- 41 NIAZI, J. H., SANG, B. I., KIM, Y. S. & GU, M. B. 2011. Global gene response in Saccharomyces
42 cerevisiae exposed to silver nanoparticles. *Appl Biochem Biotechnol*, 164, 1278-91.
- 43 NOGALES-CADENAS, R., CARMONA-SAEZ, P., VAZQUEZ, M., VICENTE, C., YANG, X., TIRADO, F.,
44 CARAZO, J. M. & PASCUAL-MONTANO, A. 2009. GeneCodis: interpreting gene lists through
45 enrichment analysis and integration of diverse biological information. *Nucleic Acids Res*, 37,
46 W317-22.
- 47 PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR.
48 *Nucleic Acids Res*, 29, e45.
- 49 PY, B. & BARRAS, F. 2010. Building Fe-S proteins: bacterial strategies. *Nat Rev Microbiol*, 8, 436-46.

- 1 ROH, J.-Y., SIM, S. J., YI, J., PARK, K., CHUNG, K. H., RYU, D.-Y. & CHOI, J. 2009. Ecotoxicity of Silver
2 Nanoparticles on the Soil Nematode *Caenorhabditis elegans* Using Functional
3 Ecotoxicogenomics. *Environmental Science & Technology*, 43, 3933-3940.
- 4 ROSENKRANZ, H. S. & CARR, H. S. 1972. Silver sulfadiazine: effect on the growth and metabolism of
5 bacteria. *Antimicrob Agents Chemother*, 2, 367-72.
- 6 ROSENKRANZ, H. S. & ROSENKRANZ, S. 1972. Silver sulfadiazine: interaction with isolated
7 deoxyribonucleic acid. *Antimicrob Agents Chemother*, 2, 373-83.
- 8 SIMPSON, J. A., CHEESEMAN, K. H., SMITH, S. E. & DEAN, R. T. 1988. Free-radical generation by
9 copper ions and hydrogen peroxide. Stimulation by Hepes buffer. *Biochem J*, 254, 519-23.
- 10 SONDI, I. & SALOPEK-SONDI, B. 2004. Silver nanopartides as antimicrobial agent: a case study on *E.*
11 *coli* as a model for Gram-negative bacteria. *J Colloid Interface Sci*, 275, 177-82.
- 12 VICKERY, H. B. & LEAVENWORTH, C. S. 1930. THE BEHAVIOR OF CYSTINE WITH SILVER SALTS. *Journal*
13 *of Biological Chemistry*, 86, 129-143.
- 14 XIU, Z. M., ZHANG, Q. B., PUPPALA, H. L., COLVIN, V. L. & ALVAREZ, P. J. 2012. Negligible partide-
15 specific antibacterial activity of silver nanoparticles. *Nano Lett*, 12, 4271-5.

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