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

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Key words

Microarray, Silver, Nanoparticle, Toxicity, Mechanism

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.

Introduction

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

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

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

silver stress than Ag(I) added as silver nitrate, even though the measured bulk solution phase Ag(I) concentration was the same. This study was restricted to a sub-set of *E. coli* Ag-responding genes but differences in the global genetic response were not investigated (McQuillan et al., 2012). In eukaryotes, including *Saccharomyces cerevisae*, a differential dissolved Ag(I)-AgNP response has been measured using microarrays (Niazi et al., 2011, Kawata et al., 2009, Roh et al., 2009, Lim et al., 2012) but to our knowledge, these experiments have never been performed in prokaryotes, which are clearly an important target group. In this study, we report the findings from whole transcriptome gene expression microarray experiments to capture the overall genetic response to (a) 142 nm AgNPs and (b) $AgNO_3$ in the Gram negative bacterium, *E. coli* K12. The response was measured at the early stage 10 minutes following silver shock at a sub-inhibitory dose to reduce background gene regulation from secondary effects including a change in growth phase. Genes that responded to both treatments are described in terms of the response to dissolved Ag(I), the common toxicant, and we report on genes that responded differentially in the two treatments reflecting differences in the mechanism of action for the two physical forms of Ag.

Methods

Materials

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

Bacterial Culture and Ag Treatment

E. coli K12 (MG1655) was received from the Coli Genetic Stock Centre and maintained on Luria agar at 37°C. All cultures were carried out in a low-salt Luria broth (LB) which was 10 g Tryptone and 5 g yeast extract in 1 L of water and pH 7.5. The salt was omitted as this improved the colloidal stability of the AgNPs and reduced precipitation of AgCl, but the medium still supported rapid growth and replication of the *E. coli*. Nanoparticle dispersion in the LB was achieved by sonicating the mixture for 2 minutes using a Soniprep 150 (MSE Instruments, London, UK). The bacterial growth curve was determined for a 100 mL culture, under aerobic conditions at 37°C with constant agitation. Viable cell numbers were measured at 30 minute intervals using the plate counting method. For AgNP treatment the dry nanopowder was dispersed in 10 mL of the LB by sonication at 10× the required concentration, then diluted to 100 mL with a log-phase culture of the *E. coli* containing 10^7 cfu/mL. For silver nitrate we used an identical procedure; log-phase cultures were diluted with fresh medium containing a 10× concentrated solution of $AgNO_3$. The exposure concentration was 40 µg/mL for AgNPs or 0.4 µg.mL for Ag(I) as $AgNO_3$. Control cultures were similarly diluted at the time of exposure and each Ag treatment was performed using quadruplicate treated/untreated control pairs.

Microarray Experiments

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

Data Analysis

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

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.

Results

The microarrays measured the global changes in gene expression in exponentially replicating (log-phase) *E. coli* after 10 minutes exposure to 142 nm AgNPs or $AgNO_3$. The nanoparticles have been characterised in our earlier study (McQuillan et al., 2012) and are composed of silver, with no surface ligand. They dissolve in the experimental medium at a rate that is linearly related to the surface area, associate directly with the cell surface and are acutely toxic to the *E. coli*. The bacteria were treated with 40 µg/mL of AgNPs or 0.4 µg.mL of Ag^+ for precisely 10 minutes in a rich medium (low salt Luria Broth). The dose did not inhibit bacterial replication in order to avoid background gene regulation associated with a change in growth phase, Figure 1.

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

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

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

Discussion

The overall genetic response to the two physical forms of silver was quite different. In total 379 genes were differentially regulated; 309 genes were only regulated by AgNPs and 70 genes were only regulated by silver nitrate. For both forms of Ag, the primary toxicant is Ag(I). For Ag added as $AgNO_3$ the labile Ag(I) can form Ag-complexes with components in the medium and labile Ag ions enter the *E. coli* through the cation selective outer membrane porin proteins. Dissolved Ag(I) is also supplied to the medium from disperse AgNPs which dissolve following oxidation of the silver surfaces. Therefore, we expect that the differential response is a result of additive toxic effects from the Ag(I) delivery mechanism. In Eukaryotes, an AgNP toxicity process independent of ion release is described for imperfections in the crystal lattice structure and highly reactive electron configurations at the NP surface (George et al., 2012, Nel et al., 2006). However, there is no evidence to support this in bacteria, which have structurally and chemically distinct membranes, in line with recent evidence that AgNPs have no anti-bacterial activity if ion release is blocked under anaerobic conditions (Xiu et al., 2012). In our earlier study, we proposed a hypothesis based on the observation that the nanoparticles associate directly with the cell surface, and dissolve on the outer membrane to create a high interfacial Ag(I) concentration, which enhances the anti-bacterial effects as a function of the labile Ag(I) concentration in the bulk solution of the medium (McQuillan et al., 2012). Accordingly in our Ag exposures, the AgNP-treated bacteria may experience membrane proximity damage and a gradient of Ag(I) from the dissolving nanoparticle acting as a point source. In contrast, exposure to silver nitrate may generate entirely different concentration gradients of $Ag(I)^+$ within the cell. AgNPs with a diameter of 12 nm, far smaller than those used in this study, have been shown to penetrate into the cell wall and enter the cytoplasm of *E. coli*, and interact directly with nucleic acids (Sondi and Salopek-Sondi, 2004, Jose Ruben and et al., 2005) which could further differentiate the AgNP-$AgNO_3$ toxicity. However, we consider this process unlikely to occur under these conditions because (1) the nanoparticles are relatively large (142 nm) and (2) sufficient membrane damage to allow entry of a particle this size would be lethal to the cell whereas the dose used in our experiments was sub-inhibitory. There are no known nanoparticle transport processes in *E. coli* or other prokaryotes that could facilitate uptake in this size range.

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

membrane damage would lead to an increase in the expression of these genes. If the cell cycle is temporarily arrested upon sudden addition of Ag, the lipid biosynthetic processes may be reduced and captured in the early phase 10 minute response. Although the overall growth profiles after exposure to both forms of Ag are equivalent, Ag(I) addeds as $AgNO_3$ has the greater lability in the medium and the initial metabolism response to AgNPs may have been delayed.

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

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

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

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

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

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

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

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

Conclusions

In conclusion, our data for the differential AgNP-$AgNO_3$ response support a growing body of evidence for a nanoparticle-specific silver ion dependenttoxicity mechanism. We propose, based upon our earlier observations of AgNPs dissolving in the medium and attaching to the cell surface (McQuillan et al., 2012), that dissolution at the cell wall produces an enhanced interfacial concentration that enters the cell. We have previously shown that nanoparticle-delivery can enhance the anti-bacterial activity of Ag(I), but this is only applicable if AgNPs are free to interact with the cell surface. Products which contain AgNPs fixed in a gel matrix (Jain et al., 2009) that cannot interact directly with bacteria may not benefit from this enhanced effect. For the overall genetic response to both physical forms of Ag the comprehensive induction of genes for the heat shock response is evidence that Ag(I) acts on protein structure, and consistent with genetic responses to silver in eukaryotic models *Drosophila melanogaster* (Ahamed et al., 2009), and *Caenorhabditis elegans* (Roh et al., 2009).The unfolded protein response has been linked with serious disease in humans and prolonged non-essential silver use should be monitored. Additional disruption occurs at iron-sulfur components leading to disruption of metabolically essential processes and could represent the critical Ag target, leading to respiratory arrest and a demand for iron, which is

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

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Figure Legends

Figure 1. Growth Plots for the *E. coli* in low salt Luria Broth. The solid line shows the growth plot for the *E. coli* in the low salt Luria Both. The bacteria were treated with Ag when the cultures reached a density of approximately 10^7 CFU/mL (2 hours). The dashed lines show the sub-inhibitory effect of the exposure concentration for AgNPs (□) and Ag(I) as $AgNO_3$ (Δ) on the post-exposure growth plot. The error bars represent the standard error of the mean for triplicate cultures.

Figure 2. GO Term Enrichment Clustering Analysis using GeneCoDis. Bar charts showing the number of genes sharing specific Gene Ontology (GO) terms that were significantly enriched in the lists of up-regulated (top) or down regulated (bottom) genes including those that were independently regulated by AgNPs or Ag(I) as $AgNO_3$. The bar labels include the term accession number.

Figure 3. Validation of microarray data by real-time PCR. The microarray gene expression data were validated by comparing the results with a set of genes (*ompF*, *cusA*, *cueO* and *soxS*) which were measured by real-time PCR using the same RNA samples. In both experiments, either (A) Ag(I) as $AgNO_3$ or (B) AgNPs linear regression analysis shows that the expression values had a strong correlation with R^2 values of 0.987 and 0.983 respectively. The units are Log 2 gene expression ratio between Ag treated and untreated control samples.

Tables

Table 1. Gene Regulation in *E. coli* Exposed to $AgNO_3$ 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 %

Table 2. Heat Shock Response Genes Induced by Silver

Gene	Function	Expression Ratio	
		$AgNO_3$	AgNPs
ipaA	Small heat shock proteins; bind and stabilise denatured polypeptides	180.16	6.44
ipaB		4.76	17.55
clpB	Disaggregation of insoluble protein aggregates	83.16	12.66
clpP	Proteolysis	2.04	3.23
dnaK	DnaK-DnaJ-GrpE (DJE) complex; chaperone for protein folding; protein disaggregation; regulation of the heat shock response	19.32	6.62
dnaJ		3.46	2.06
grpE		4.96	4.25
groS	GroEL complex; chaperone for protein folding; protein re-folding	9.93	5.69
groL		9.44	5.44
htpG	Protein folding; homologue to mammalian HSP90	11.33	5.97
htpX	Protease; degradation of denatured polypeptides	8.94	5.47
hslJ	Heat shock locus proteins	*	5.91
hslO		3.62	3.37
hslR		2.90	2.62
hslU		2.36	2.28
hslV		2.33	2.72
lon	DNA-binding ATP-dependent protease	2.85	3.11
idhA	NAD-linked fermentative lactate dehydrogensase; associated with heat stress	3.01	2.48
hflK	Putative proteases; associated with heat stress	2.88	2.31
hflX		*	2.41
rrmJ	Ribosome associated methyltransferase; associated with heat stress	2.76	3.12
cpxR	CpxAR regulon; responds to protein unfolding in the periplasm; responds to Cu^+	*	2.80
ppiA		*	2.67
dsbA		*	2.57

* failed expression cut-off or confidence filters

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

Gene	Function	Expression Ratio	
		$AgNO_3$	AgNPs
iscA	Fe-S assembly complex	4.11	2.09
iscU		3.65	2.06
iscS		3.14	*
iscR	Regulatory protein for *iscSUA*	7.99	2.68
sufA	Fe-S assembly complex	62.29	16.26
sufB		30.58	10.82
sufC		20.58	11.97
sufD		18.06	7.83

* failed expression cut-off or confidence filters

Table 4. Fur Regulon Genes Induced by Silver

Gene	Function	Expression Ratio	
		$AgNO_3$	AgNPs
entA	Biosynthesis of the enterobactin siderophore	146.78	9.19
entB		116.39	10.65
entC		161.96	4.91
entD		6.82	4.69
entE		77.22	12.95
entF		53.56	15.07
ybdB		160.24	7.49
Cir	Outer Membrane Fe^{3+}/ligand receptors	386.65	4.44
Fiu		41.44	3.32
fepA	Uptake of Fe^{3+}/enterobactin	47.48	12.21
fepB		55.40	2.87
fepC		24.10	*
fepD		20.76	*
fepG		27.12	*
fecR	Uptake of ferric citrate	29.91	2.05
fecI		35.64	2.27
fecA		5.21	*
fecB		2.99	*
fhuA	Uptake of ferrichrome	14.38	*
fhuC		5.62	*
fhuE		20.93	16.07
fhuF		19.91	*
tonB	Inner membrane complex; energises the outer membrane Fe^{3+}/ligand transporters	2.43	*
exbB		4.19	*
exbD		4.03	*

* failed expression cut-off or confidence filters

Table 5. Sulfate Transport and Assimilation Genes Induced by Silver

Gene	Function	Expression Ratio	
		$AgNO_3$	AgNPs
cysA		16.19	6.50
cysC		4.29	5.94
cysD		4.49	7.00
cysH		13.66	6.97
cysI	Sulfate assimilation and biosynthesis of cysteine	8.49	7.83
cysK		3.70	2.49
cysN		23.75	6.74
cysP		2.02	2.72
cysW		5.54	5.22

Table 6. Copper Homoestasis Genes Induced by Silver

Gene	Function	Expression Ratio	
		$AgNO_3$	AgNPs
copA	P-type ATPase; copper transporter	47.09	13.71
cueO	Periplasmic cuprous oxidase	320.45	36.72
cusC	RND-protein driven cation/proton exchanger; may transport Cu^+ and Ag^+; *cusF* encodes a small Ag^+/Cu^+ binding protein	72.48	40.54
cusF		376.39	54.08
cusB		99.98	26.90
cusA		50.32	20.72
cusR	Two-component regulatory system; regulates *cusCFBA*	2.53	5.11
cusS		*	2.83

* failed expression cut-off or confidence filters

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