Experimental evolution of bacteria exposed to sub-inhibitory concentrations of silver nanoparticles

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Declaration

I would specially like to thank my supervisor Michael Gillings for constant guidance throughout the project. I would also like to thank Amy Asher, Louise Chow and Tim Ghaly for constructive discussion during laboratory work. All other research described in this report is my own original work. I hereby certify that this thesis has not been submitted for a Master's Degree at any other University or institution.

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Abstract

The rise of antibiotic resistant pathogens emphasises the need to develop new antimicrobials to treat bacterial infections. Silver nanoparticles are a new antimicrobial which harness the intrinsic toxicity of silver and make it more effective as an antimicrobial agent. Silver nanoparticles pollution is increasing because nanosilver is commonly added to many consumer goods. Pollution will generate sub-inhibitory concentrations in some environmental compartments which may facilitate the appearance and spread of resistance to silver, through mutation, recombination and lateral gene transfer events. The frequency of each of these events is increased by the SOS response. Therefore, if silver nanoparticles induce the SOS response, widespread resistance to silver nanoparticles becomes likely. If resistance becomes widespread, we will have compromised an antimicrobial that could prove useful in a post-antibiotic era.

In this series of experiments, I aimed to induce the SOS response in *Escherichia coli* and *Acinetobacter baumannii* by exposing each species to sub-inhibitory concentrations of silver nanoparticles over a prolonged period. There was no evidence that the SOS response was induced, but the assay did not seem to work, as ciprofloxacin, a known inducer of the SOS response, also induced no change. However, several lines exhibited silver resistance. This resistance cannot be due to acquisition of known resistance mechanisms, such as the *sil* operon, since there was no opportunity for lateral gene transfer during these experiments. Consequently, it seems likely that mutations that confer resistance have occurred within some lineages. Identification of the loci involved will require genome sequencing of the resistant isolates.

Keywords: Silver nanoparticles, bacterial resistance, mutation, SOS response, experimental evolution, *Escherichia coli, Acinetobacter baumannii*

Introduction

The post-antibiotic era may be the greatest medical challenge society will face in the 21st century (Brown & Wright 2016). Increasing numbers of human pathogens now cause infections that are difficult to control due to their accumulation of antibiotic resistance genes (Berdy, 2012). Development of new antibiotics is slow and expensive, so there is a growing interest in other agents that exhibit antimicrobial activity. Silver has long been used in health care due to its strong antimicrobial activity (Lansdown, 2006).

In the last decade, the antimicrobial effect of silver has been improved with the development of silver nanoparticles (Wong, 2010; Aruguete *et al.* 2013). Silver nanoparticles are nanoscale (1-100nm) particles of silver, which are often combined with carbon and silicon compounds (Foldbjerg *et al.* 2015). Silver nanoparticles are a stronger antimicrobial agent than silver ions alone, and for this reason they have begun to replace silver in catheters, prostheses and bone cements in clinical settings (Monteiro *et al.* 2009; Potera, 2012). Nano silver is also being used in bandages and salves for burn victims (Jones *et al.*, 2003).

There is potential for silver nanoparticles to be used in conjunction with antibiotics to combat multidrug resistant bacteria and slow the spread of resistance (Shahverdi *et al.* 2007; Chen *et al.* 2014; McSahn *et al.* 2014). Silver nanoparticles are included in numerous commercial products due to their antimicrobial properties (Guo *et al.* 2015). Of the 1827 currently listed products that use nanotechnology, 442 have silver nanoparticles (CPI, 2016). The widespread use of silver nanoparticles will inevitably result in pollution, and increase unintentional exposure of microbes to silver nanoparticles.

The impact that silver nanoparticles will have in the environment, particularly in aquatic settings, has been examined, but few definite conclusions have been drawn (Gil-Allue *et al.* 2014). It is widely agreed that it will affect ecosystems and will increase human exposure to silver nanoparticles (Fabrega *et al.* 2010). The risks of exposure to humans are also poorly understood. Silver nanoparticles are thought to be harmful if inhaled, and ingestion is likely to be harmful to the human microbiota (Qadros & Marr, 2010; Das *et al.* 2015).

At a minimum, bacteria that inhabit aquatic ecosystems, agricultural land, hospitals and human microbiota will inevitably be exposed to varying concentrations of silver nanoparticles. At low concentrations, the silver nanoparticles will not kill bacteria, but may promote resistance via SOS responses that increase general rates of mutation, recombination and lateral transfer (Andersson &

Hughes, 2012). Sub-inhibitory concentrations may also be sufficient to select resistant lineages when these arise.

Interesting parallels can be drawn between antibiotic pollution and silver nanoparticle pollution. Although less well studied, it seems that low concentrations of silver nanoparticles may have the same impact on bacterial evolution as low concentrations of antibiotics (Chow *et al.* 2015; Graves *et al.* 2015). To that end, pollution may impair the long term viability of silver nanoparticles as a potent antimicrobial agent. To maximise the efficacy of silver nanoparticles in clinical medicine, greater care might be needed in their usage and disposal.

Antimicrobial Activity of Silver Nanoparticles

The toxicity mechanisms of silver, against microbes in particular, have been well studied (Sondi & Sondi, 2004; Rai *et al.* 2008). Silver ions can bind to proteins containing phosphorous and sulphur, can attach to thiol groups, and can bind directly to nucleosides (Fig. 1; Mijnendonckx *et al.* 2013). Each of these actions disrupts the function of the cell and can lead to cell death (Asharani *et al.* 2009). All of these toxicity pathways are reliant on the presence of silver ions, which can be released from a compound or nanoparticle through interaction with oxygen (Feng *et al.* 2000). Although silver is a non-specific antimicrobial agent, differences in toxicity have been observed between target species (Kim *et al.* 2007; Arnaout *et al.* 2012). Consequently, it can be inferred that membrane structure is intrinsically linked with the toxicity mechanisms mentioned above.

In binding to sulphur-containing membrane proteins, silver ions block respiration and electron transfer by displacing hydrogen ions (Dibrov *et al.* 2002; Mijnendonckx *et al.* 2013). Blockage of membrane channels, in particular, alters the flow of protons and disrupts the membrane, which allows silver ions to enter the cell (Feng *et al.* 2000; Mijnendonckx *et al.* 2013). Once inside the cell, silver ions interact with and condense DNA, which blocks transcription, limiting the ability of the cell to maintain function (Rai *et al.* 2008). Interaction with thiol groups, notably of cysteine, disrupts protein folding and can inactivate many of the cell's enzymes (Mijnendonckx *et al.* 2013). In aerobic species, these effects combine to cause the production of reactive oxygen species which cause further damage to proteins (Mijnendonckx *et al.* 2013). *E. coli* strains that had mutations in DNA repair genes caused by oxidative lesion were less resistant to silver nanoparticles (Radzig *et al.* 2012).

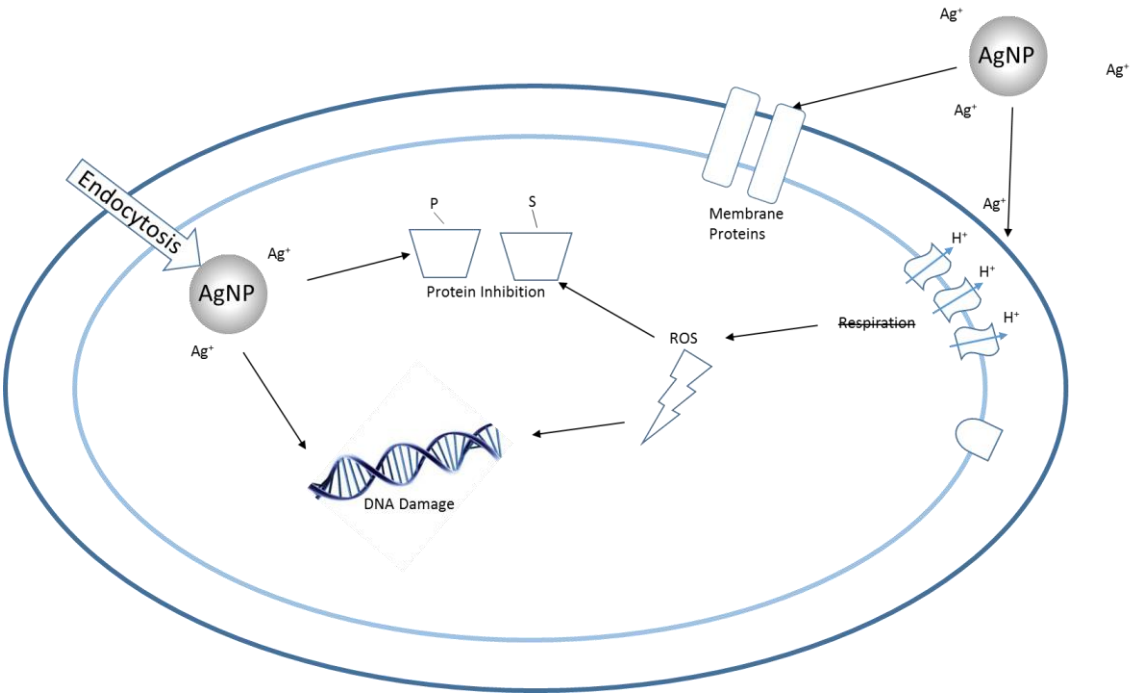

Figure 1: Toxicity mechanisms of silver nanoparticles. Toxicity of silver nanoparticles (AgNP) is mainly due to silver ions (Ag^+). Silver ions can bind to membrane proteins and shut down the electron transport chain. This inhibits respiration and produces reactive oxygen species (ROS). ROS can damage both DNA and proteins within the cell. AgNPs and Ag^+ can also bind to DNA and proteins, which condenses cellular components and disrupts function.

Much of the research conducted into the toxicity mechanisms of silver was conducted before the development of silver nanoparticles. In aerobic conditions silver nanoparticles release silver ions, however it has been found that nanoparticles are more toxic than would be expected if their antimicrobial action were due to ions alone (Yin *et al.* 2011; Gil-Allie *et al.* 2014; Gitipour *et al.* 2016). There is conjecture about whether this increased toxicity is due to a novel toxicity mechanism of silver nanoparticles, or if they facilitate a more efficient delivery of silver ions due to their physicochemical properties (Morones *et al.* 2005; El-Nour *et al.* 2010; Xiu *et al.* 2012; Helmlinger *et al.* 2016). Silver nanoparticles can also inhibit biofilm formation, which then increases the vulnerability of bacteria to many antimicrobials (Radzig *et al.* 2012; Perez-Diaz *et al.* 2015).

Potential factors for the increased toxicity in silver nanoparticles include: size (Choi *et al.* 2008), solution pH and organic matter (Fabrega *et al.* 2009), surface area (Perez-Diaz *et al.* 2014), and shape, surface coating and surface charge (Xiu *et al.* 2012). However it was argued by Xiu *et al.* (2012) that each of these may affect silver ion release rather than having a directly toxic effect. Surface coating is added to some nanoparticles to prolong colloidal stability. This causes

agglomerations to occur less frequently, and the benefits of nanoparticles (size, surface area) are maintained (Fabrega *et al.* 2010).

The influence of the size of the nanoparticle on toxicity is not clear. There is some evidence that the nanoparticle interacts with the membrane of bacteria and contributes to antimicrobial activity (Sotriou & Pratsinis, 2010). These authors concluded that larger nanoparticles exerted some antimicrobial mechanism in themselves, which did not occur when smaller nanoparticles were used. In contrast, smaller nanoparticles have also been correlated with increased toxicity, possibly because a high surface area influences silver ion release. Therefore different nanoparticles may have different minimal inhibitory concentrations, this being the lowest concentration that will inhibit cell growth (Perez-Diaz *et al.* 2015). Structural composition may consequently have influences on the environmental impact of silver nanoparticles.

The effect of silver nanoparticles on humans is unclear (El-Nour *et al.* 2010). Testing in murine models has yielded conflicting results. No toxicity was noted in a drug that contained silver nanoparticles. In fact, some aspects of mouse physiology were enhanced by the drug (Daniel *et al.* 2010). However, in human cell lines endoplasmic stress was caused by silver nanoparticles, and cells from the lung, liver and kidney were particularly sensitive to exposure (Huo *et al.* 2015). Although silver nanoparticles are now used in a multitude of products, it remains unclear whether their use and consumption might have a detrimental impact on human health.

Environmental Pollution

The increasing prevalence of silver nanoparticles in commercial products inevitably means that a gradient of pollution will occur around human activity. Basic silver nanoparticles can be generated from silver objects, including cutlery and jewellery, in humid air and water (Glover *et al.* 2011). As there is a high antimicrobial activity of silver nanoparticles at concentrations as low as 0.1μg/L, even small concentrations will exert negative impacts on microbial communities (Fabrega *et al.* 2009; Kumari *et al.* 2014). Currently, silver nanoparticles account for only 15% of silver pollution, but this number is predicted to increase steadily as more consumer goods incorporate silver nanoparticles (Perello 2013). Modelling has suggested that silver pollution may exceed the predicted no effect level, especially in aquatic sediments (Blaser *et al.* 2008). The model suggests that a no observed effect concentration of 320 μg/L should be established in toxicological studies (Blaser *et al.* 2008). Reported pollution levels for silver in aquatic environments are well below the minimal inhibitory concentrations reported in toxicity studies. However, effluent from industrialised

areas can reach 5 ppm, which may be sufficient to inhibit some species of bacteria (Lasko and Hurst, 1999).

During waste treatment, approximately 85% of silver nanoparticles are deposited into sludge, mostly in the form of Ag_2S or as $AgCl_x$ in saline environments (Kaegi *et al.* 2011; Wang *et al.* 2016). Sludge is often used as fertiliser, and even at low concentrations, silver nanoparticles have a negative impact on some crop species and are likely to have a negative impact on soil microbiota (Hirsch, 1997; Ratte, 1998; Qian *et al.* 2013; Hoppe *et al.* 2014). Of the nanoparticles that remain in the water stream, most settle into the sediment, but some remain in suspension and may enter marine and estuarine ecosystems (Chinnapongse *et al.* 2011).

The impact that silver nanoparticles will have on aquatic species is unclear. Minimal bioaccumulation and biomagnification was noted for several silver species, but some impact was noted in relation to the formation of fish eggs (Ratte *et al.* 1998; Fabrega *et al.* 2010). It is likely that human consumption of silver nanoparticles will occur, but research suggests that the amount consumed will be minimal. Even at low concentrations, consumption may disturb the balance of the microbiota (Das *et al.* 2014; Das *et al.* 2015). Entrance into the digestive tract may also create a concentration gradient of silver nanoparticles. At points along the gradient of pollution, in the environment or the intestine, the concentration of silver nanoparticles will drop below the minimal inhibitory concentration, which may provide ideal conditions for the development of silver resistance, much the same as has occurred in the development of antibiotic resistance in environmental strains of some bacteria (Andersson & Hughes, 2012).

Based on the results of the second chapter of this thesis, the reported toxicity of silver nanoparticles in the literature may not be applicable to aquatic environments. Most aquatic systems will have a lower concentration of protein than media in the laboratory. Therefore, reported minimum inhibitory concentrations will over-estimate the amount of silver needed to trigger a reaction in an aquatic environment. Conversely, the minimum inhibitory concentration reported in the second chapter likely underestimates the environmental toxicity, as the silver nanoparticles will bind to protein and possibly other compounds, and their bioavailability will be decreased. To predict a no effect concentration for silver nanoparticles, future tests should simulate environmental conditions for toxicity studies.

Parallels to Antibiotic Resistance

The spread of antibiotic resistance has rendered antibiotics ineffective against some strains of human pathogens (Alanis, 2005). Misuse and abuse of antibiotics has contributed to the rapidity of the spread and accumulation of resistance genes (Martinez, 2008; Berdy, 2012; Larsson, 2015). Silver nanoparticles represent a viable alternate antimicrobial agent, and as such we should use it in ways that minimise the likelihood that resistance will develop.

One of the strategies to slow the spread of resistance is to delay the widespread use of new antimicrobials (Courvalin 2008). Therefore, with new antimicrobial agents, greater regulation of usage will help extend their viability in the clinical setting. Precautions for silver nanoparticle use are currently not in place. The large number of silver nanoparticle products means that pollution is already occurring unnecessarily (Benn & Westerhoff, 2008; Kaegi *et al.* 2010). Much like antibiotics, silver nanoparticles are being included in products for their antimicrobial qualities, when there is no necessity for antimicrobial activity. The misuse of silver nanoparticles will increase the amount of environmental pollution. This in turn might generate optimal concentrations that promote the evolution and spread of resistance (Hermsen *et al.* 2012). If silver pollution continues, many of the patterns observed for antibiotic resistance will also apply to silver nanoparticles (Andersson & Hughes, 2012).

The Evolution of Resistance to Silver

Resistance arises via three main mechanisms: mutation, recombination and lateral gene transfer (Chow *et al.* 2015). The presence of antibiotics at constant sub-inhibitory levels is known to increase the rate of each of these mechanisms, and also selects for individuals that have acquired resistance phenotypes via any one of these mechanisms (Andersson & Hughes, 2012; Chow *et al.* 2015). Spatial heterogeneity in the concentration of selective agents may create an environment analogous to a source-sink population, in terms of movement of resistance elements (Hermsen *et al.* 2012). Similar patterns have been noted for heavy metal pollution (Nies, 1998; Teitzel & Parsek, 2003). The similarities between the behaviour of these toxic agents suggest that the spread of silver nanoparticle resistance will follow similar trends.

For some time it was believed that bacteria would be unable to evolve resistance to silver nanoparticles, due to the broad range of targets for their antimicrobial action (Kim *et al.* 2007). Resistance to such broad scale actions were thought to require multiple independent mutations in the same organism, thus decreasing the likelihood of resistance arising (Hermsen *et al.* 2012).

However, over a decade ago it was found that bacteria had developed resistance to silver that was present in the effluent of hospitals and photographic factories (Gupta & Silver, 1998; Davis *et al.* 2005). Plasmid systems were found that contained up to nine genes associated with silver resistance (the *sil* operon), most of which encoded efflux pumps and membrane proteins that bound silver ions (Gupta & Silver, 1998). These proteins stopped silver ions binding to sulfhydryl proteins, therefore respiration was not inhibited (Mijendonckx *et al.* 2013). Randall *et al.* (2015) found that endogenous resistance to silver ions could develop in six days in *E. coli*. However, *P. aeruginosa, A. baumannii, C. freundii* and *S. sonnei* did not develop resistance after 42 days of exposure. Currently, these silver resistance genes do not seem to be prevalent in known human pathogens (Woods *et al.* 2008; Loh *et al.* 2009). It is unclear if genes from the *sil* operon will also confer resistance to silver nanoparticles. The majority of silver nanoparticle toxicity seems to stem from the action of ions alone and therefore the *sil* operon may confer resistance. If the increased toxicity of silver nanoparticles is due to increased local concentration of silver ions rather than a novel mechanism, then it may also be assumed that the *sil* operon will confer resistance.

Possible mechanisms for silver nanoparticle resistance include: methylation or demethylation of DNA, efflux pumps, extracellular or intracellular sequestration, alteration of target sites or decreased permeability. Each of these resistance mechanisms has been noted in heavy metal resistant strains (Hobman & Crossman, 2014). Metal ion resistance genes are often found on multidrug resistance elements and silver is no exception (McHugh *et al.* 1975). Clinical strains of silver ion-resistant bacteria have recently been discovered, which contained genes homologous to known heavy metal resistant elements (Finley *et al.* 2015). This raises the possibility of co-resistance and co-selection of resistance elements by silver nanoparticles, other metal ions and possibly other antimicrobials (Pal *et al*. 2015. Pollution with any antimicrobial may increase the prevalence of resistance genes to any other antimicrobial and with each new antimicrobial, resistance may arise more rapidly. Therefore, management strategies such as antimicrobial cycling or modified antimicrobials might not slow the spread of resistance significantly.

It has yet to be established whether sub inhibitory levels of silver nanoparticles can trigger the SOS response. The SOS response is a relatively conserved stress response which is mounted in response to DNA damage. The SOS response is well categorised in *E. coli*, but homologous systems are found in many other species, even though the genes involved in the SOS response vary between species (Galhardo *et al.* 2007; Baharoglu & Mazel 2014).

When single stranded DNA is abundant in the cell, expression of the RecA protein is increased. The RecA protein derepresses the LexA inhibitor and activates the expression of approximately 40

genes. Three of these genes code for proteins that eventually make up DNA polymerase II, IV and V (Galhardo *et al.* 2007). DNA Pol IV and V are part of the Y-family DNA polymerases, which are low fidelity, specialised DNA polymerases involved in translesion synthesis (Sale *et al.* 2012). This allows a stalled replication fork to continue and therefore the cell may survive. However the mutation rate is increased, because of the poor proof reading activity of these enzymes (Tompkins *et al*. 2003).

Therefore, the SOS response affects DNA mutation rates after the introduction of a stress, by stimulating translesion synthesis and disrupting DNA mismatch repair (Ennis *et al.* 1994; Baharoglu & Mazel, 2014; Culyba *et al.* 2015). During SOS, cells enter a state of hypermutation, which increases the chance of a fitter mutant arising (Blazquez 2003). The SOS response also increases the frequency of lateral gene transfer and recombination events (Ubeda *et al.* 2005). Through these mechanisms, the SOS response is known to increase the generation and spread of resistance. The SOS response has been implicated in the generation of antibiotic resistance, and is triggered at sub-inhibitory concentrations (Chow *et al.* 2015).

Since silver nanoparticles may damage DNA and proteins involved in translation, the SOS response might be mounted in response to sub-lethal exposure. Therefore, it is possible that low levels of silver nanoparticles in effluent streams will increase the regularity of the SOS response and thus increase the generation and uptake of resistance genes.

There is concern that antimicrobial agents are selecting for bacterial strains that have a greater evolvability (Gillings & Stokes, 2012). That is, individuals that have a lower capacity for horizontal gene transfer and situational mutation and recombination are less likely to survive in the presence of a selective agent. Strains that have evolved antibiotic resistance may be better at developing resistance to other antimicrobial agents. It is possible that each new antimicrobial drug will become ineffective more rapidly as hyper-adaptable strains develop. The implications of this may influence the long term viability of silver nanoparticles as antimicrobial agents, and may indicate that care should be taken in the usage of silver nanoparticles.

In this experiment, I aim to examine whether sub-inhibitory concentrations of silver nanoparticles can induce the SOS response in *E. coli* and *A. baumannii*. If there is evidence of the SOS response, some strains may also have developed increased resistance to silver nanoparticles.

Methods

Bacterial Isolates

Two species were used in this experiment: *Escherichia coli*, and *Acinetobacter baumannii*. Strain information is listed in Table 1. Both species are increasingly prevalent human pathogens, and multidrug resistance strains have been discovered (Johnson *et al.* 2010; Dijkshoorn *et al.* 2007). *E. coli* is a gut bacterium which can cause food poisoning and urinary tract infection. *A. baumannii* is a common nosocomial pathogen which infects open wounds, catheters, the respiratory tract and urinary tract. A *recA* mutant and wild type strain of each species were generously provided by Professor Ian Paulsen of Macquarie University. Samples were maintained on lysogeny agar (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1.5% Agar) at 37°C. Single colonies were suspended in a solution of equal parts 50% glycerol and minimal salts and stored at -80°C for future analysis.

Table 1: List of bacterial strains used in this work

Species	**Strain**	**Genome**	**Reference**
E. coli	BW25113 (Wild type)	Genbank: CP009273	Grenier *et al.* 2014
	JW2669-1 (*recA* mutant)	Keio Collection	Baba *et al.* 2006
A. baumannii	AB5075 (wild type)	Genbank: PRJNA77021	Zurawski et al. 2012
	Kr130904p01q137 (*recA* mutant)	N/A	N/A

Silver Nanoparticles

Five different elemental silver nanoparticle products were tested for antimicrobial activity. Four of these products were citrate stabilised and suspended in 2M sodium citrate at 0.02 mg/mL. They were: 10 nm silver nanoparticles (Sigma-Aldrich), 10 nm silver nanospheres (Nanocompsix), 100nm silver nanoparticles (Sigma-Aldrich) and <200nm silver nanoparticles (Nanocomposix).

As expected, the smaller nanoparticles were the most toxic. Subsequently, 10nm PVP coated silver nanoparticles (Nanocomposix) were preferred, partly due to their decreased agglomeration caused by steric hindrance rather than electrostatic repulsion. This meant that the nanoparticles were less

likely to agglomerate when exposed to salts and amino acids present in culture media. PVP coated silver nanoparticles are also generally found to be more toxic than citrate coated silver nanoparticles (Badawy *et al.* 2011; Van Dong *et al.* 2012). All nanosilver products were provided by Dr Megan Osmond of CSIRO. Silver nanoparticles were stored in the dark at 4°C and were vortexed prior to usage. The PVP coated silver nanoparticles were imaged under a TEM and measured using ImageJ (Abramoff *et al.* 2004) for size verification. The nanoparticles were also analysed via XPS to determine amount of ionisation after 5 months of storage.

Determination of Minimum Inhibitory Concentrations

The minimum inhibitory concentration (MIC) was determined for each strain against each nanosilver product, following the protocol of Weigand *et al.* (2008). MIC assays were performed in microtiter trays containing LB and M9 minimal media (0.7% Na_2HPO_4, 0.03% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.4% dextrose or sodium succinate, 10^{-3} M $MgS0_4$ and 10^{-4} M $CaCl_2$; Sambrook & Russell, 2001). The silver nanoparticles were serially diluted so that a wide range of concentrations was tested with each assay. Silver concentrations began at 40 mg/L in LB and 60 ug/L in M9 minimal medium. A growth control, which contained no antimicrobial, and a sterility control were included in each assay. Each well was inoculated with approximately 20000 cells, determined via a spread plate viable cell count. Plates were incubated on a shaker at 37°C for 18h. Bacterial growth was determined by optical density in a Pherastar FS (BMG Biotech) at 540nm. The MIC was recorded as the lowest concentration where growth was completely inhibited. An MIC assay was also performed for sodium citrate as the citrate stabilised nanoparticles were suspended in 2M sodium citrate. Sodium citrate generated an inhibition curve that was similar to the 10nm citrate coated silver nanoparticles, and may have confounded results. Therefore PVP coated silver nanoparticles, which are suspended in water, were used for the final MIC analyses and subsequent experiments. MICs were reassessed after 25 generations of exposure to silver nanoparticles. The Students t-test was used to detect significant changes in resistance.

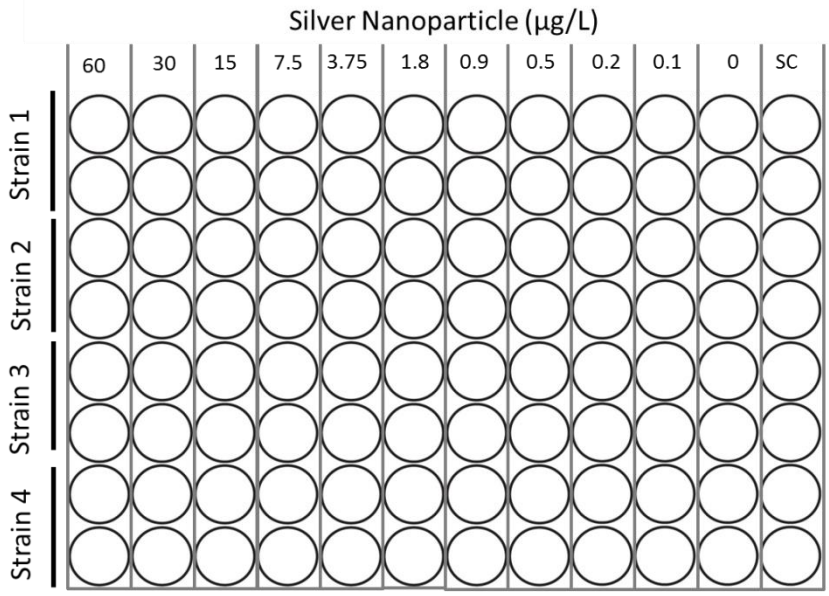

Figure 2: Schematic of the MIC assay in a 96 well microtiter plate. The concentration of silver nanoparticles was halved each column. Each bacterial strain was assayed twice, and the average used for analysis. Labels SC = sterility control (uninoculated).

Silver in Agar

Silver nanoparticles were added to LB agar at MIC and twice MIC concentrations to confirm toxicity in the solid state. All four strains grew at twice MIC. Examination of the agar under an Olympus FLUOVIEW 1000 confocal laser scanning microscope suggested the silver nanoparticles were not uniformly distributed, causing inconsistent toxicity. The microscopy revealed agglomerations of silver nanoparticles around impurities in the agar (See supplementary information). Because of the potential difficulty in controlling nanoparticle concentration and delivery, subsequent experiments were performed in liquid media.

Experimental Evolution

Bacteria were exposed to sub-inhibitory concentrations of silver nanoparticles in liquid broth using serial passaging. Cultures were revived by streaking onto LB agar with overnight incubation at 37°C. Single colonies were suspended in 0.1M potassium phosphate buffer (pH 7.4), to a concentration of approximately 2 x 10^6 cells/mL. This suspension was used to inoculate liquid medium in microtiter trays. Microtiter trays were prepared as follows: 500µL aliquots of M9 minimal media were dispensed into 12 wells of 24 well microtiter plates. Alternate wells were left empty to limit the potential for well to well cross contamination. The final experimental set up had 12 aliquots of medium. Four contained M9 minimal media only, to serve as controls, and eight were supplemented with 1/10^{th} the MIC for silver nanoparticles, this being a final concentration of 3µg/L

10nm PVP coated silver nanoparticles (Nanocomposix). The wells were then inoculated with 10µL of bacterial suspension.

The microtiter plates were incubated at 37°C on a shaker for 16-24hr. A loop-full of each liquid culture then was streaked onto individual LB agar plates and incubated at 37°C overnight, when a single, well isolated colony was selected and suspended in phosphate buffer, as above. This cycle of processes was labelled as one generation. In total, 25 generations were run using this method. At every five generations, a loop full of culture was put into M9 medium made up to 20% v/v glycerol for storage at -80°C. As a positive control for SOS induction, both *E. coli* strains were exposed to $1/10^{th}$ the MIC for the antibiotic ciprofloxacin, cycling as above for five generations.

DNA Extractions

DNA was extracted every five generations using a bead-beating method (Yeates & Gillings, 1998; Gillings, 2014). 100µL of glycerol stock was added to 800µL of CLS-TC buffer (MP Biomedicals) in a Fast-prep tube. The tubes were shaken in a FastPrep FP120 machine (BIO 101 Savant) at 5.5m/s for 30s then centrifuged (Eppendorf 5417C) at 14000rpm for 5 min. 700µL of supernatant was collected and added to 700µL of to a DNA binding matrix solution. The binding matrix contained two parts 6 mol L^{-1} guanidinium thiocyanate to one part Binding Matrix (BIO 101, consisting of glassmilk). The solutions were mixed on the rotor for 5 min, microcentrifuged for 10s at 14000rpm and the supernatant was discarded. Pellets were resuspended in 800µL salt ethanol wash solution (SEWS: 70% ethanol, 100 mmol L^{-1} sodium acetate) and left to sit for 2 mins. Samples were then spun for 10s at 14000rpm, then the supernatant was discarded and the pellets were left to air dry for 5 mins. The pellet was resuspended in 200µL of TE (pH 8.0), and then the mixture was pelleted by microcentrifugation at 14000rpm for 3min. 160µL of the DNA containing-supernatant was stored at -20°C.

Repetitive Element PCRs and recA PCR

Repetitive element PCR was performed every five generations to generate a DNA fingerprint, to check for contamination and assess gross changes to the genome. 9µl of GeneReleaser™ (Bioventures Inc.) and 1µl of DNA were mixed in a 0.5mL PCR strip tube and microwaved on high for 7 min. Tubes were then heated at 80°C for 5 min in an Eppendorf ep gradient S Mastercycler (Eppendorf AG, Hamburg, Germany). For each sample, 40 µl of reaction solution was prepared which contained: 25 µl of GoTaq® White (Promega), 13.5 µl of sterile H_2O, 0.5µl RNase (1 mg/ml), and 1µL 50µM of the appropriate primer(s). Different primer combinations, including BOX (5' CTACGGCAAGGCGACGCTGACG); ERIC (ERIC1R: 5'

ATGTAAGCTCCTGGGGATTCAC and ERIC2 5’ AAGTAAGTGACTGGGGTGAGC); and M13F (5’ GTTTTCCCAGTCACGAC) primers were tested for each strain.

A gradient PCR was performed to ascertain the ideal annealing temperature for the primers. PCR conditions are listed in Table 2. To perform electrophoresis, 7 µl of each PCR product was loaded with 5 µl of Bromophenol loading dye onto a 2% agarose TBE (90mM TRIS-borate and 2mM EDTA) gel and run in TBE at 110V for 150 mins. The gels were stained with GelRed™ (Biotium Inc.) and visualised under UV light. A 100bp ladder was added with every gel. The BOX reaction was preferentially used as it generated the clearest and most complex fingerprints for analysis.

Repetitive element DNA fingerprints were indistinguishable between the *recA* mutants and the wild types, demonstrating that the strains were essentially isogenic except for the *recA* mutation. A PCR which targeted *recA* was used to discriminate between the RecA+ and RecA- strains of each species. Primers were designed that targeted the RecA gene in: *E. coli* (ECrecaF: 5’ TGGTAAAGGCTCCATCATGC and ECrecaR: 5’ AGTTTCTGCTACGCCTTCGC) and *A. baumannii* (ABrecaF: 5’ GGTGATAACACTGTTCAAGC and ABrecaR: 5’ TTCATCTTCAATTTGAACTGC). The PCR solutions were prepared as described above and PCR conditions are listed in Table 2. As each set of primers was species specific they also served as a secondary cross-species contamination check. Assays were designed so that the mutants differed

Table 2: Thermal cycling programs used to generate the BOX fingerprints and to amplify the *recA* gene

PCR	Thermal Cycle
BOX for *E.coli*	94°C 3min 94°C 30s 52°C 30s 72°C 8min (35 cycles) 72°C 15min 4°C hold
BOX for *A.baumannii*	94°C 3min 94°C 30s 52°C 30s 72°C 8min (35 cycles) 72°C 15min 4°C hold
E.coli recA PCR	94°C 3min 94°C 30s 60°C 30s 72°C 90s (35 cycles) 72°C 5min 4°C hold
A.baumannii recA PCR	94°C 3min 94°C 30s 56°C 30s 72°C 90s (35 cycles) 72°C 5min 4°C hold

from the wild types in the size of the PCR product for *A. baumannii* or in presence/absence of an amplification product for *E. coli*.

DNA Banding Analysis

The degree of genomic change was assessed using the images of the BOX PCR fingerprints. Fingerprints from generation 25 were compared to a control profile from generation 0. Differences were scored to calculate a similarity coefficient (F) using the formula of Nei & Li (1979): $F=2N_{xy} / (N_x + N_y)$. Where N_x and N_y are the number of bands in the lane x and lane y and N_{xy} is the number of bands shared by lane x and lane y. Identical banding patterns will have a similarity coefficient of 1. The closer F is to 0, the greater the difference between the samples. The fingerprints were scored blind by an individual uninvolved with the experimental process. F statistics were graphed on a scatterplot for visual comparison.

Results

Silver nanoparticles

The silver nanoparticles used in the experiments reported here were approximately 7.8 ± 3.8 nm in diameter, meaning they are some of the smallest nanoparticles commercially available, and therefore should have reasonably high toxicity compared to the larger silver nanoparticles. XPS revealed that most of the silver remained in the form of silver nanoparticles after five months of storage.

Initial MIC

When tested in M9 minimal media (See Chapter 2) the MIC of all four strains remained constant, from trial to trial. MICs ranged from 15 to 60µg/L (Fig 3). In contrast, MICs in LB medium were significantly higher and less reproducible. Consequently, M9 media was used for all experimental evolution assays. RecA mutants were consistently more sensitive to antimicrobials and grew more slowly during the toxicity tests and during the experimental evolution trial. Taking the difference between RecA mutant and wild type into account, *E. coli* exhibited approximately two times the MIC value of *A. baumannii*.

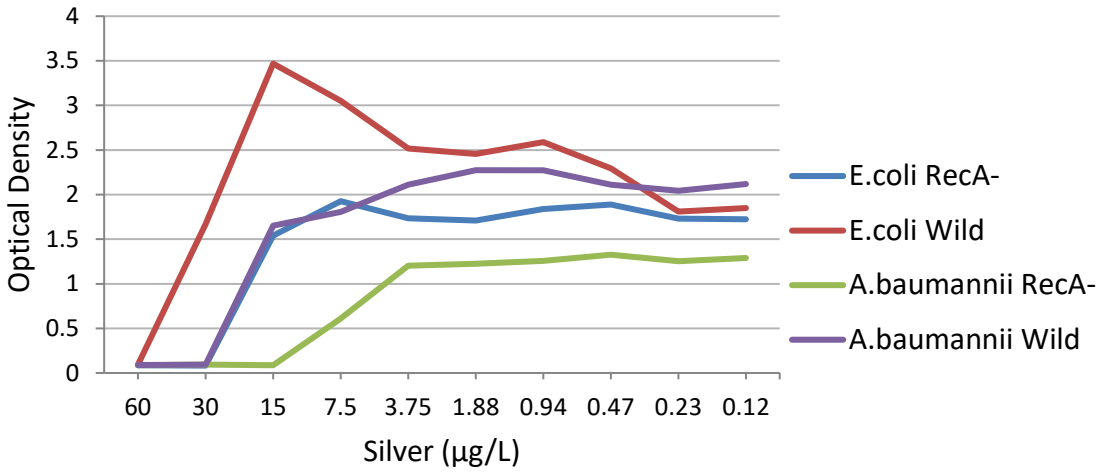

Figure 3: MIC assay of *E. coli* and *A. baumannii* strains at generation 0. Each data point is the mean of two replicates.

DNA fingerprint analysis

BOX, ERIC and M13F PCRs were used to detect genome changes that may have been induced by exposure to silver nanoparticles. The principle behind these PCRs is described in Gillings & Holley (1997). Briefly, the primers have reproducible binding sites within every genome which will amplify a large number of sequences, which can then be used to generate a complex DNA fingerprint. Mutations change the binding sites of the primers, and therefore changes can be seen in the fingerprints. Such changes are also generated by indels, inversions, transpositions and recombination events. The BOX primer was used in the majority of analyses as it generated the most complex DNA fingerprints. An example of the fingerprints generated from experimental and control lines of *E. coli* at generation 25 is shown in Figure 4. No changes were visible in the DNA fingerprints of *E. coli* during tests performed at any generation. There were also no differences between the wild-type and RecA mutants.

In comparison, similar experimental evolution experiments using ciprofloxacin, a known inducer of the SOS response, generated significant fingerprinting differences in as few as five generations in ciprofloxacin treatment lines (Chow *et al.* 2015). However, during this experiment, using liquid media and the protocol given above, ciprofloxacin treatment lines exhibited no detectable genomic changes in *E. coli* after five generations (See supplementary information).

Significant polymorphisms were detected in the fingerprints of *A. baumannii* for every line (Fig 5 & 6), however there was no significant difference between the wild-types and the RecA mutants ($p = 0.355$) or between control and experimental lines ($p = 0.516$).

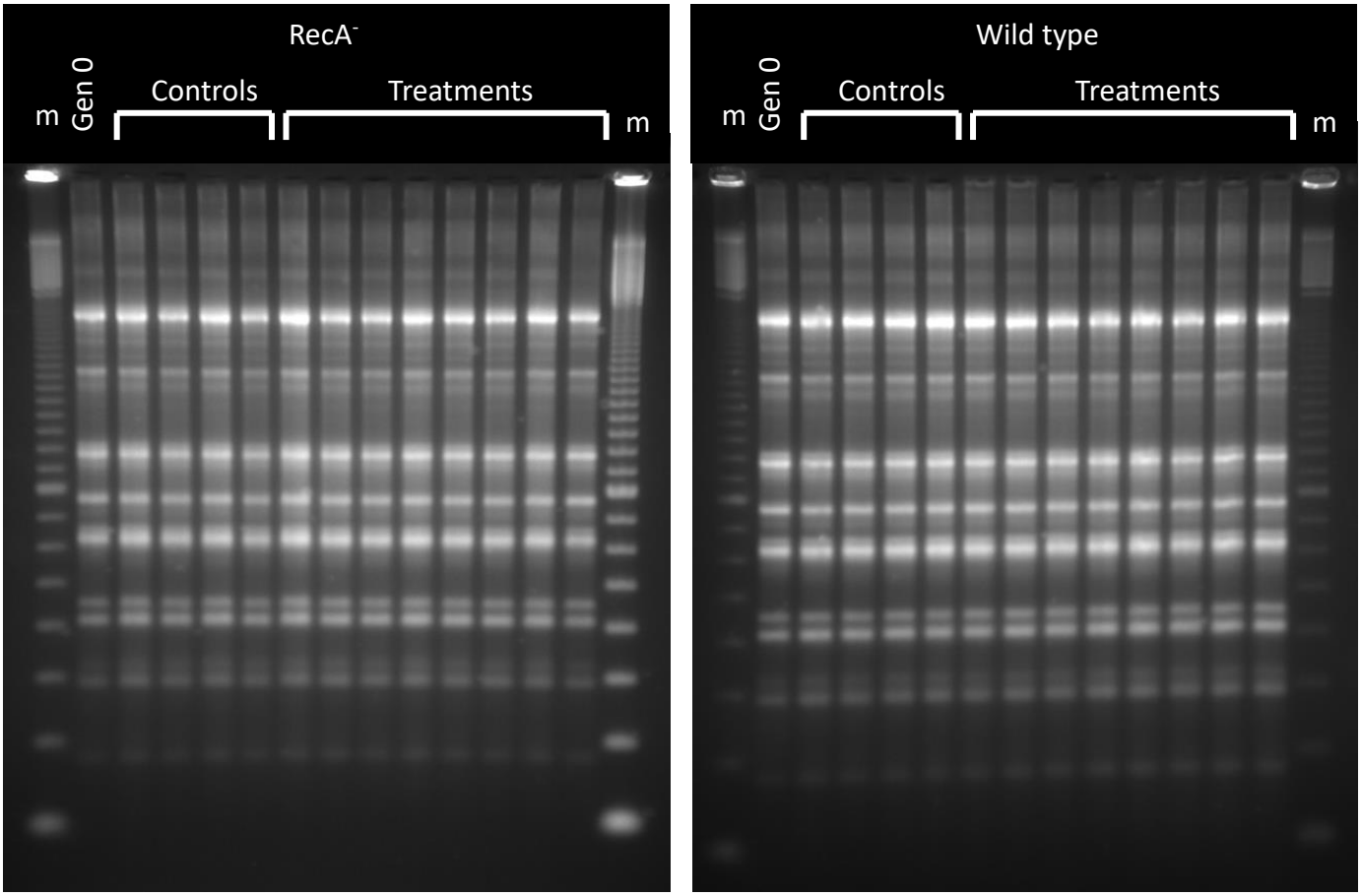

Figure 4: BOX PCR DNA fingerprints for *E. coli* at generation 25. Labels: m = 100bp ladder, Gen 0 = generation 0 comparison.

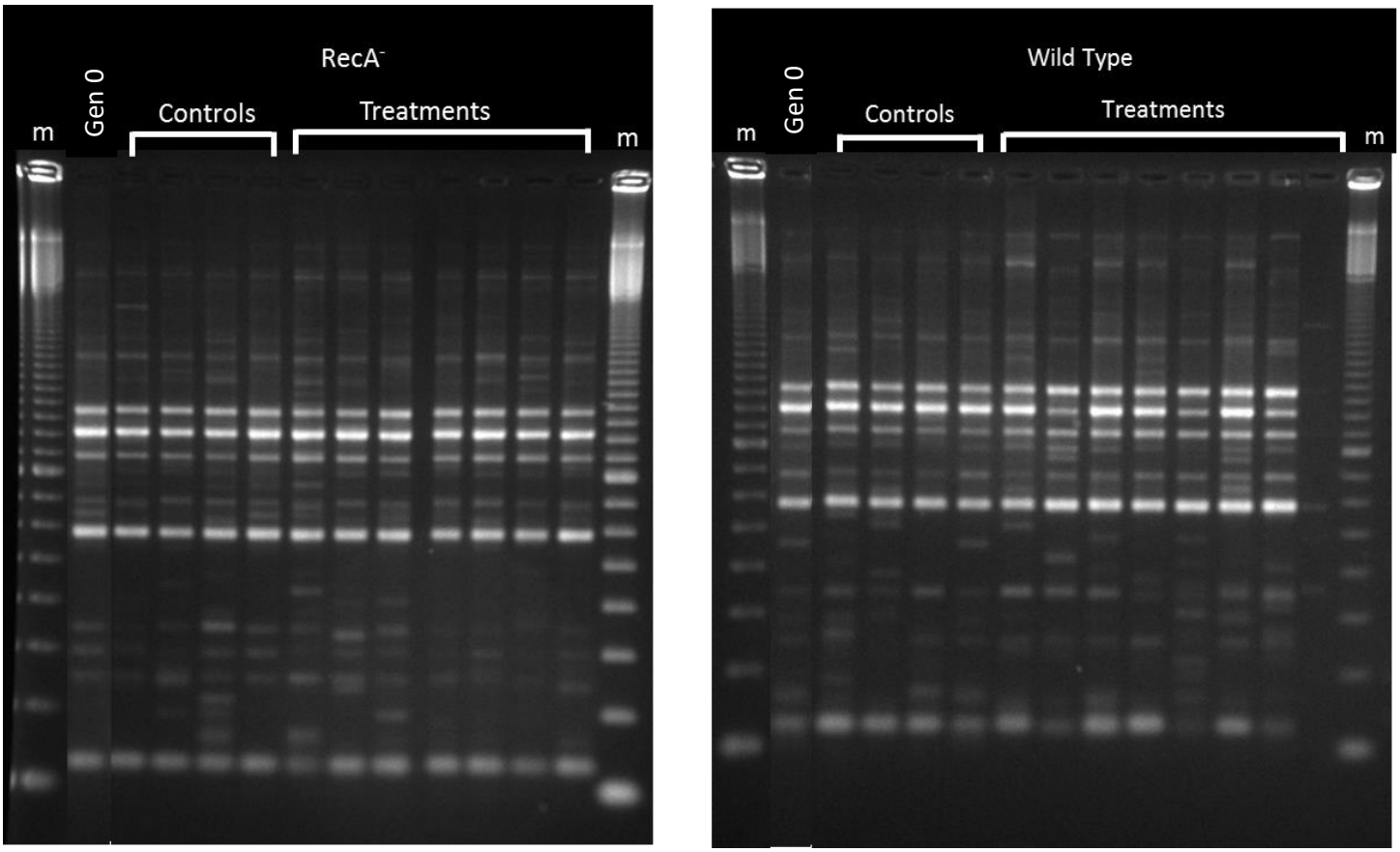

Figure 5: BOX PCR DNA fingerprints for *A. baumannii* at generation 25. Treatment 8 of the wild type was omitted from analysis. m = 100bp ladder.

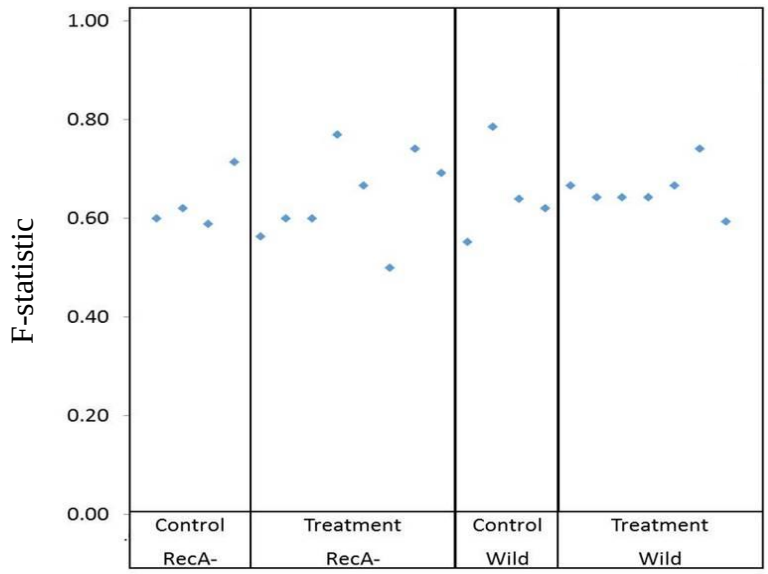

Figure 6: Similarity coefficients (F) of BOX fingerprints of *A. baumannii* at generation 25 compared to generation 0.

Changes in MIC

The MIC of each experimental line was assessed after 25 generations of exposure to silver nanoparticles to examine for changes in silver resistance compared to controls (Fig. 7 & 8). The only lines to exhibit significantly increased resistance were the *E. coli recA* mutants, as shown by a t-test on the optical density readings from both replicates of each sample (DF = 17, T = -2.92, p = 0.009). Four lines of *E. coli recA* mutants exhibited a two to three fold increase in MIC. The other three strains exhibited minor changes in MIC but there was no significant difference between treatments and controls.

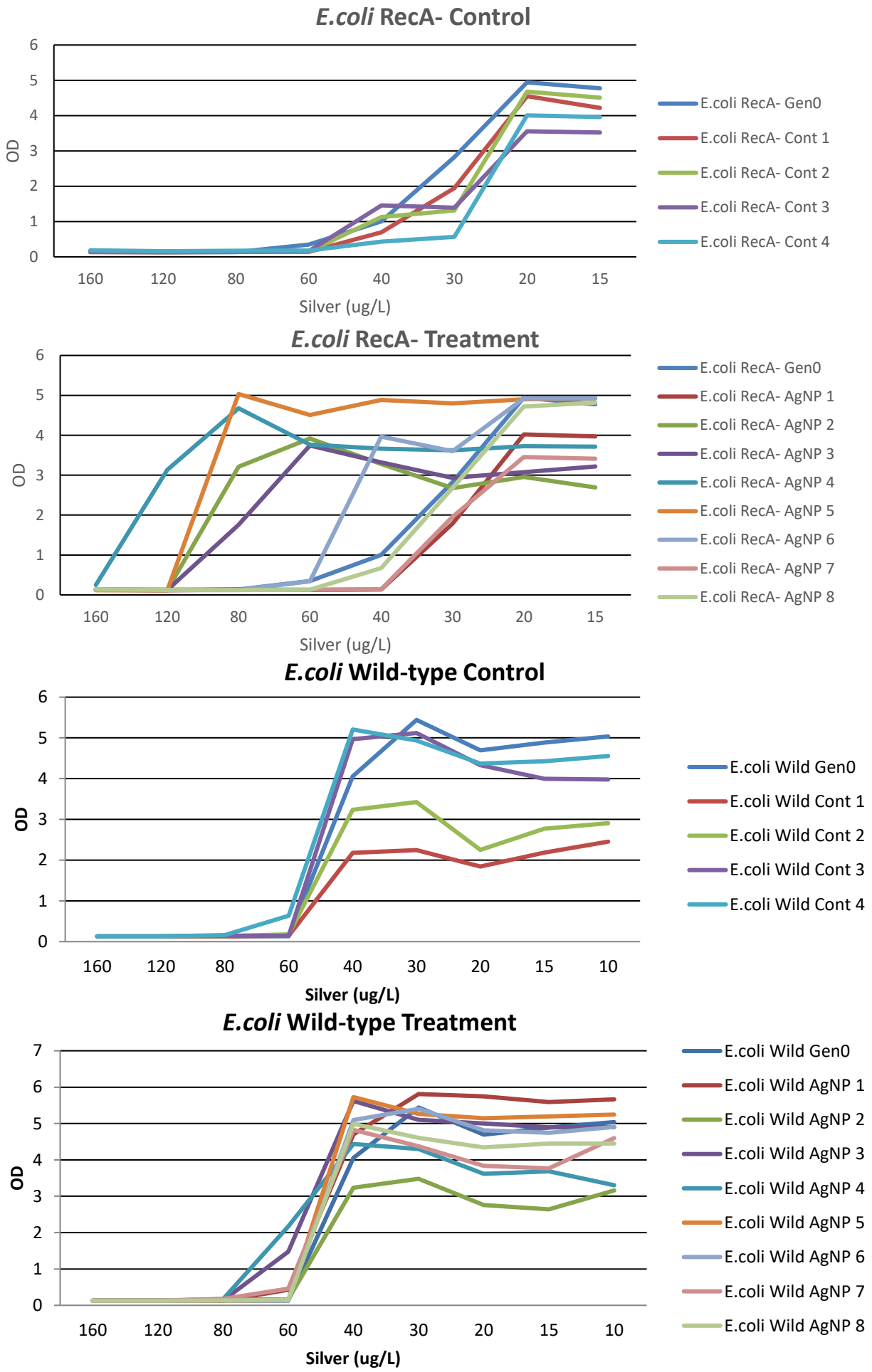

Figure 7: Inhibition curves for all *E. coli* isolates at generation 25. The MIC was recorded as the lowest concentration to inhibit all visible growth. Gen0 was included for comparison. OD = optical density

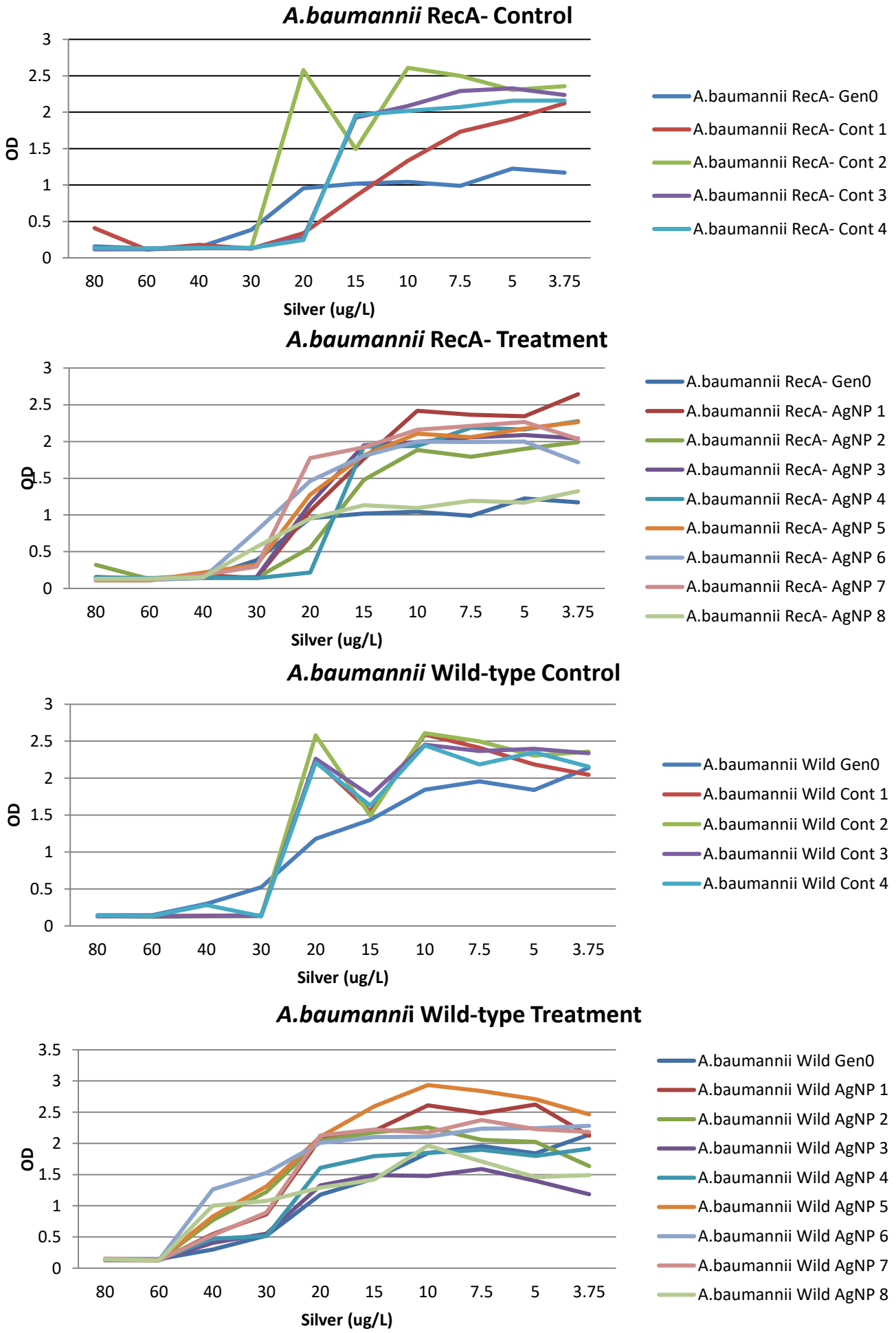

Figure 8: Inhibition curves for all *A. baumannii* isolates at generation 25. The MIC was recorded as the lowest concentration to inhibit all visible growth. Gen0 was included for comparison. OD = optical density

Discussion

In this series of experiments, two significant clinical pathogens were exposed to sub inhibitory concentrations of silver nanoparticles to test if this could select for resistance. *E. coli* and *A. baumannii* were serially passaged in liquid culture for 25 'generations'. At the end of this series of transfers, the experimental lines were DNA fingerprinted to confirm strain identity, and to detect gross rearrangements, recombination events, transpositions and mutations. This test was performed to assess the ability of silver nanoparticles to induce the SOS response, which in turn is known to increase the frequencies of all these genomic changes. To attribute any observed effects to the SOS response, isogenic *recA* minus lines were similarly tested, since such lines cannot mount an SOS response.

No evidence that silver induces the SOS response

The *E. coli* lines showed no evidence of mutation or recombination in the DNA fingerprints. *A. baumannii* showed fingerprint changes in every strain, but there was no significant difference between experimental and control treatments, or between *recA* mutants and wild type strains. Therefore, there was no indication that the SOS response was triggered by exposure to sub-inhibitory concentrations of silver nanoparticles. Either there was not a significant amount of single-stranded DNA being generated by exposure to silver nanoparticles, or the experiment did not create the conditions necessary to promote and detect the SOS response.

These bacteria were exposed to sub-inhibitory silver concentrations for approximately 600 hours over the course of the experiment, which should be sufficient time to induce an SOS response, as exposure to ciprofloxacin for approximately 100 hours is known to cause significant genome changes that are readily detectable with the DNA fingerprinting method used here (Chow *et al.* 2015).

E. coli and *A. baumannii* were exposed to silver nanoparticles in liquid culture, and subsequent growth on LB agar plates to generate single colonies for the next generation was in the absence of antimicrobial. One possibility is that periodic exposure to an antimicrobial does not have the same impact as continuous growth and selection in the presence of an antimicrobial. This may explain why exposure to ciprofloxacin for five generations caused no observable change in the DNA fingerprints. The relationship between concentration and exposure time is known to affect the expression of stress regulated genes (Holder *et al.* 2013). A concentration of $1/10^{th}$ MIC or exposure for 18-24 hours each generation, may not result in the expression of genes that could alter the genome and generate resistance.

The DNA fingerprinting method has potential limitations for detecting genomic changes. The BOX fingerprints are an insensitive tool for detecting genome change, as only 14-20kb (~0.3%) of the genome is being sampled in any one assay. The error prone DNA polymerases target specific regions in the genome, which may lie outside the binding locations of the BOX primer (Sale *et al.* 2012). However, to detect no genome change in eight lines of wild type *E. coli* and no significant effects of silver in eight lines of wild type *A. baumannii*, suggests that SOS induced hypermutation was not present in the experimental lines.

Theoretically, ssDNA could have arisen in the bacteria via the influence of either silver ions or ROS (Kohanski *et al.* 2010). Silver ions may bind to DNA and provide a physical obstacle to replication, or they may bind to proteins involved in replication and thereby stall the replication fork. Silver ions can disrupt the cell wall, in a mechanism similar to ß-Lactams, and may trigger the SOS response by this process (Miller *et al.* 2004). ROS can create double strand breaks that would also stall the replication fork (Smith & Walker, 1998). Silver ions and ROS also affect other components of the cell and, perhaps, the impact that they have on DNA is comparatively minor (Poole, 2012). Therefore, other stress responses, such as oxidative stress response, may be mounted, rather than the SOS response. Other stress responses can upregulate some proteins that are involved in the SOS response, so it is possible that some indications of SOS activity may be due to starvation, heat shock or peroxide stress (Farr & Kogoma, 1991; Arce *et al.* 2013). As silver nanoparticles impact most aspects of cell physiology, the tolerance for silver nanoparticles is very low and there may be a critical threshold for silver nanoparticle tolerance. DNA damage would be one of the last impacts of silver nanoparticles, by which time the cell may be severely compromised and in terminal decline. Therefore silver nanoparticles may be triggering the SOS response in some cells, but these cells will not survive to pass on mutations that would result from error prone replication.

When the silver nanoparticles are introduced to the media, they will start to agglomerate around the bacteria, which will decrease their colloidal stability and bioavailability. The condensed bodies may then sink to the bottom of the media and have little impact on the other cells in the culture, which explains why some cells survive. However, bacteria are known to be sponges for silver and, once the cell dies, the silver is leached back into the culture and may impact other cells (Wakshlak *et al.* 2015). If so, there would be a strong relationship between the number of cells added and silver toxicity, however, my experiment showed little evidence for this.

Resistance to silver nanoparticles

At the close of the experimental evolution experiments, all lines were tested for elevated resistance to silver nanoparticles. Four experimental lines showed two to threefold increases in MIC. All these

lines were founded from the *recA* mutant *E. coli* line JW2669-1. There was no significant increase in MIC for the wild type *E. coli* lines or for any line of *A. baumannii*.

It is unusual that some lines of *E. coli* RecA mutants developed resistance, while the wild types did not. The RecA gene in line JW2669-1was replaced with a kanamycin resistance gene, otherwise the two strains were isogenic (Baba *et al.* 2006), as confirmed by the identity of the BOX fingerprints between *recA* mutant (JW2669-1) and wild type (BW25113) strains. If mutations in the genome of *E. coli* JW2669-1 could give rise to lines with increased MICs, it seems likely that similar mutations could have occurred in the wild type mutants, but this was not observed. One difference between the mutant and wild-type strain was their initial sensitivity to silver nanoparticles. The RecA mutant lines had a lower initial MIC, and consequently the concentration of silver nanoparticles used in the experiment was closer to their tolerance threshold. This concentration may have applied more selective pressure on the *recA* minus lines, and thus fixed resistance mutations more effectively.

In some species, RecA mutants are inhibited in their ability to become resistant, and are more susceptible to other stresses (Aranda *et al.* 2011). This suggests the SOS response is important for a cell's long term survival (van der Veen *et al.* 2010). SOS-induced DNA polymerases in *E. coli* convey a fitness advantage over knockout mutants, and can be transiently expressed in the absence of a stress during stationary stage (Yeiser *et al.* 2002). This may explain why the RecA mutants were more sensitive to silver nanoparticles initially.

Recently, Graves *et al.* (2015) used an *E. coli* strain that was free of silver resistance elements to show that resistance to silver nanoparticles can develop rapidly in the presence of silver nanoparticles. By generation 300, mutations had accumulated that enabled *E. coli* to survive in concentrations nearly five times higher than they had previously (Graves *et al.* 2015). Although the concentrations of silver nanoparticles used were higher than would currently be expected in aquatic environments, this experiment acts as a proof of concept. That is, at sub inhibitory levels, silver nanoparticles favour mutants that display increased tolerance to silver nanoparticles.

When multiple mutations are required for resistance, hypermutation, recombination and lateral gene transfer become very important for a population's survival (Eliopoulos & Blázquez, 2003; Tompkins *et al.* 2003). In the resistant strains of *E. coli* generated during the current experiment, there was no indication that recombination or hypermutation had occurred, and there was no opportunity for lateral gene transfer. Therefore, the resistance exhibited by *E. coli* might be a multidrug resistance mechanism, such as an efflux pump, rather than a mechanism specific to silver nanoparticles (Su *et al.* 2012; Du *et al.* 2014).

Many antimicrobials exist in the environment at sufficient concentrations to generate and select resistance. Heavy metals (cadmium, chromium, selenium and uranium) can select and upregulate genes that protect against oxidative stress, DNA repair enzymes, membrane proteins, multidrug efflux pumps and transporters (Hu *et al.* 2005). Commercial biocides, such as aldehydes and ammonium compounds, induce mutations in specific areas of the genome, which confer multidrug resistance (Weeber *et al.* 2015). Disinfectant by-products can select for multidrug resistance, by selecting for mutations in a multidrug efflux pump and specific resistance genes (Lv *et al.* 2014; Li *et al.* 2016). Triclosan and triclocarban are broad scale antimicrobials that can significantly increase resistance to a host of antibiotics at environmentally relevant concentrations (Carey & McNamara 2015; Carey *et al.* 2015; Yueh & Tukey, 2016). Silver nanoparticles also increase the abundance of resistance genes in aquatic environments (Ma *et al.* 2016). Pollution with any of these biocides can induce coselection of resistance elements (Baker-Austin *et al.* 2006; Seiler & Berendonk 2012). As all of these are prevalent pollutants in aquatic systems, the mixture of biocides may create complex, novel combinations of genes that improve the survivability of bacteria to any cytotoxic stress, including silver nanoparticles (Beaber et al; 2003; Webber *et al.* 2015).

Lateral gene transfer and recombination can create large mobile elements, which will be positively selected by most of the antimicrobials listed above (Summers, 2016). Mobile elements can contain genes that improve the metabolism, maintenance and evolvability of the organism (Zhang *et al.* 2006; Gillings, 2007; MacLean *et al.* 2010). These genes confer a constant advantage and will be selected in the absence of antimicrobial stress. Areas of pollution can act as a reservoir of resistance genes for pathogens (Salyers & Shoemaker, 2016). When new antimicrobials are developed, areas with a high abundance of resistant strains could be able to quickly adapt resistance mechanisms to the new agent. Therefore, the time it takes for pathogens to become resistant to novel antimicrobials may be continually decreasing. If pollution with the antimicrobial can be minimised, resistance will take longer to originate and spread. This highlights the importance of regulation in the use of new antimicrobials, such as silver nanoparticles.

An increasing number of antimicrobials are being found that trigger the SOS response (Rodriguez *et al.* 2016). Although there was no evidence of silver nanoparticles causing the SOS response in either species during this experiment, resistance to silver nanoparticles could still arise via the SOS response. Antibiotics and the antimicrobials listed above are abundant in aquatic environments and can trigger the SOS response (Maiques *et al.* 2006; Kohanski *et al.* 2010). Unlike antibiotics, silver nanoparticles do not biodegrade, and may persist in suspension or sediment for extended

periods (Richter *et al.* 2015). Silver could impose a selective pressure on bacteria in these systems and resistance may then arise.

Conclusion

Silver nanoparticles can be a potent weapon in clinical medicine and help control pathogens which have become resistant to antibiotics. With their increasing use, environmental pollution is inevitable no matter what precautions are taken (Boholm *et al.* 2014). However, if silver nanoparticles are limited to uses which definitely require antimicrobial activity, pollution will be minimised. These precautions were not taken with antibiotics and this, in turn, may have helped generate and fix resistance in pathogens. As a consequence, one of the most important medical breakthroughs in history, may become ineffective less than 100 years after its development. Drawing parallels to antibiotic resistance may help in better managing silver nanoparticles and other new antimicrobial agents. If trends in usage continue, low levels of silver nanoparticle will become a permanent feature of many ecosystems, which I have shown can potentiate the rapid development of resistance through mutation only. The presence of silver nanoparticles may then provide a selective force and increase the probability of resistance genes becoming widespread. If resistance to silver nanoparticles becomes widespread another antimicrobial agent will be rendered ineffectual.

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Protein concentration of media impacts toxicity of silver nanoparticles

Abstract

Determination of the toxicity of an antimicrobial agent is a critical consideration for management of bacterial infections and for calculation of dosage. In the face of growing resistance to conventional antibiotics, silver nanoparticles are being suggested as one option for controlling microbial growth. Factors that influence toxicity of silver nanoparticles have been extensively researched, but one factor that is often not considered is the protein content of media used for susceptibility testing. A review of existing literature showed that there is substantial variation between toxicity results, and that this seems to be associated with the media used for testing. It is known that protein concentration affects the colloidal stability of silver nanoparticles and therefore their bioavailability and toxicity. This phenomenon may be important to consider when determining the concentration of silver nanoparticles needed to control bacterial spread and infection, both in medicine and industry. Here we test the effects of exogenous protein on determination of the MIC for silver nanoparticles, and show that the addition of protein can significantly affect the estimated MIC value.

Introduction

Silver has a long history as a broad spectrum antimicrobial. In Roman times, it was used to keep water, eating utensils and food containers sterile (Clement & Jarrett 1994). For the next 2000 years it was one of the most useful antimicrobials, as it had few side effects (Wong & Liu 2010). The development of antibiotics in the 1930s caused a reduction in usage of silver in medicine, but forms of ionic silver are still being used in bandages, bone cements, catheters and medical instruments (Panyala *et al.* 2008). Various species of silver have been used to treat intestinal diseases, syphilis and gonorrhoea (Alexander 2009). Since the 1950s, colloidal silver has gained popularity as a generalised treatment, but the advertised benefits of colloidal silver tend to be overstated (White *et al.* 2003).

Silver nanoparticles are a major advancement in enhancing the antimicrobial efficacy of silver (Foldbjerg *et al.* 2015). At the nanoscale (1-100nm), the silver particles have novel physiochemical properties, which increase the toxicity of silver and therefore increase silver's usefulness as an antimicrobial. As nanotechnology advances, certain new aspects of silver nanoparticles may be improved further, including; toxicity, colloidal stability, specificity and ionisation rate. Silver

nanoparticles are already used in an array of medical devices and consumer products. Silver nanoparticles have the potential to replace some antibiotics in the post-antibiotic era (Alanis 2005).

Misuse and abuse of antibiotics contributed to the spread of antibiotic resistance, so with any new antimicrobial, plans should be in place to minimise the generation of resistance. Determination of dosage is one consideration for a management plan, as sublethal concentrations create the conditions for resistance to arise (Graves *et al.* 2015). There has been a significant amount of research dedicated to determining the toxicity of different silver nanoparticles towards different bacteria, but there is little similarity in the protocols that have been used. A wide range of minimal inhibitory concentrations (MICs) have been reported in the literature (Table 1). Different bacterial species and different silver nanoparticle formulations will have different MIC values to a certain extent (Khaydarov *et al.* 2009), but reported values can vary over two orders of magnitude. The composition of the media used for testing is not standardized, and this factor is frequently not considered when toxicity is being measured (Kittler *et al.* 2010).

In the previous chapter, it seemed that certain characteristics of LB medium inferred with silver nanoparticles toxicity. In this experiment, I examined the influence of protein concentration on the toxicity of silver nanoparticles. Silver nanoparticles tend to agglomerate around amino acids and so become less bioavailable. Therefore, in the presence of protein, silver nanoparticles might show decreased toxicity against microbes. This experiment suggests that a standardised method for determining the MIC of silver nanoparticles should be introduced.

Table 1: Comparison of published values for silver nanoparticle MICs in different broths

Article	Species	MIC	AgNPs Characteristics	Media
Khaydarov et al. 2009	*E. coli*	3mg/L	7nm	Nutrient Broth
	E. coli	34mg/L	70nm	Nutrient Broth
	S. aureus	2mg/L	7nm	Nutrient Broth
	S. aureus	25mg/L	70nm	Nutrient Broth
	B. subtilis	19mg/L	7nm	Nutrient Broth
Lkhagvajav et al. 2011	*S. aureus*	4mg/L	20-45nm	Muller Hinton Broth
	P. aeruginosa	2mg/L	20-45nm	Muller Hinton Broth
	B. subtilis	3mg/L	20-45nm	Muller Hinton Broth
	C. albicans	3mg/L	20-45nm	Muller Hinton Broth
	S. typhimurium	3mg/L	20-45nm	Muller Hinton Broth
	E. coli	3mg/L	20-45nm	Muller Hinton Broth
	K. pneumoniae	4mg/L	20-45nm	Muller Hinton Broth
Radzig *et al*. 2013	*E. coli AB1157*	0.5mg/L	8.3 +- 1.9nm	Difco nutrient broth
	P. aeruginosa	8mg/L	8.3 +- 1.9nm	Difco nutrient broth
	P. chlororaphis	8mg/L	8.3 +- 1.9nm	Difco nutrient broth
	S. proteamaculans	2mg/L	8.3 +- 1.9nm	Difco nutrient broth
Du *et al.* 2008	*E. coli*	3mg/L	Chitosan loaded with Ag	Muller Hinton Broth
	S. choleraesuis	3mg/L	Chitosan loaded with Ag	Muller Hinton Broth
	S. aureus	6mg/L	Chitosan loaded with Ag	Muller Hinton Broth
Kumar & Mamidyala 2011	*B. subtilis MTCC 121b*	16mg/L	8-24nm (Avg 13)	Muller Hinton Broth
	S. aureus MLS 16	8mg/L	8-24nm (Avg 13)	Muller Hinton Broth
	S. aureus MTCC 96	32mg/L	8-24nm (Avg 13)	Muller Hinton Broth
	M. luteus MTCC 2470	8mg/L	8-24nm (Avg 13)	Muller Hinton Broth
	E. coli MTCC 739	32mg/L	8-24nm (Avg 13)	Muller Hinton Broth
	P. aeruginosa MTCC 2453	16mg/L	8-24nm (Avg 13)	Muller Hinton Broth
	K. planticola MTCC 2453	16mg/L	8-24nm (Avg 13)	Muller Hinton Broth
Yang *et al.* 2013	*P. stutzeri*	4mg/L	35nm	Difco nutrient broth
	A. vinelandii	12mg/L	35nm	Modified Burks medium
Li et al. 2011	*S. aureus*	5mg/L	-	Mueller Hinton Broth
Kumari et al. 2014	*E. coli ATCC 13534*	1mg/L	30-40nm	Saline solution
	E. coli ATCC 25922	1mg/L	30-40nm	Saline solution
	S. aureus	1mg/L	30-40nm	Saline solution
Sondi & Salopek-Sondi 2004	*E. coli*	60mg/L	12nm	LB agar
Graves et al. 2015	*E. coli*	0.5mg/L	10nm	Davis Minimal Broth

Methods

Bacteria

Escherichia coli BW25113 was used in this study. The genome has been sequenced (Genbank: CP009273, Grenier *et al.* 2014) and contains no known silver resistance genes. Samples were cultured in M9 minimal media with 0.2% w/v dextrose as a carbon source (Sambrook & Russell, 2001).

Antimicrobials

10nm PVP coated and citrate coated silver nanoparticles (Nanocomposix) were tested for toxicity against *E. coli*. The PVP coated nanoparticles generally gave more consistent toxicity and were suspended in water, as opposed to sodium citrate which may confound results. Ciprofloxacin was used as a control.

MIC

Silver and ciprofloxacin MIC assays were set up as described in the previous chapter, following established protocols (Wiegand et al., 2008). In addition, different concentrations of bovine serum albumin (BSA) were added to each row of the microtiter plate (See Fig. 1). Therefore, every well had a unique combination of silver nanoparticle and BSA. Concentrations of silver nanoparticle ranged from 500 μg/L to 1 μg/L. The highest concentration of BSA used was 500 mg/L. Wells were then inoculated with approximately 20000 cells. Plates were placed on a shaking incubator maintained at 37°C for 24hrs. Bacterial growth was measured by optical density in a Pherastar FS (BMG Biotech) at 540nm. The MIC was recorded as the lowest concentration where growth was inhibited. The MIC was then plotted against BSA concentration to generate a curve.

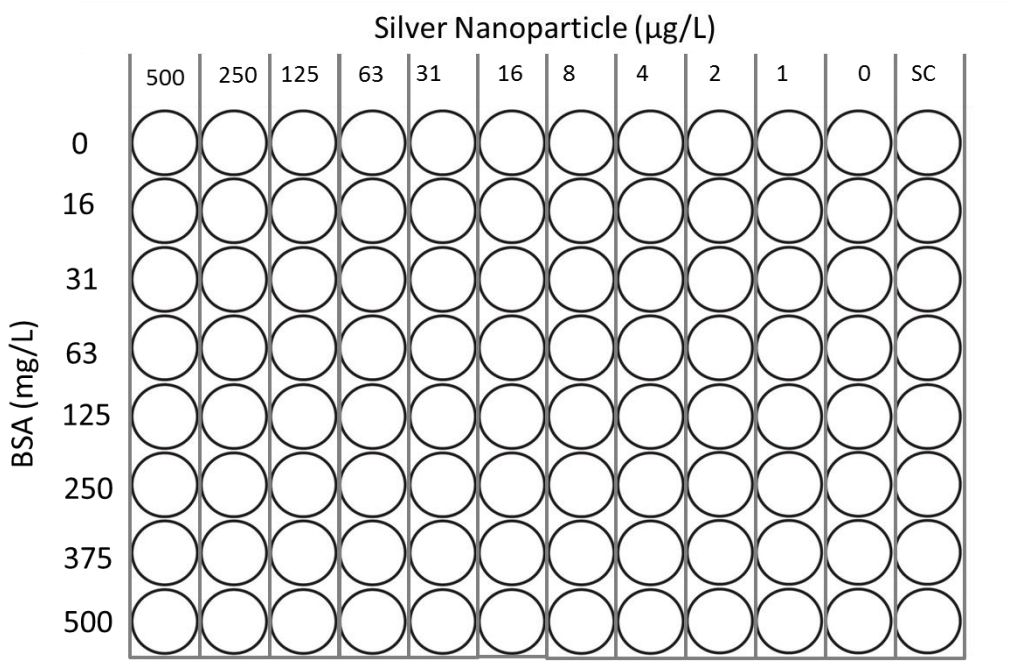

Figure 1: Schematic of the MIC assay in a 96 well microtiter plate. The concentration of silver nanoparticles was halved each column. The same concentration of bovine serum albumin was added to each row, so that each well contained a unique BSA to AgNP ratio. SC = sterility control.

MIC Analyses

Optical density readings were averaged for five assays of silver nanoparticle toxicity. A line graph was generated to detect the MIC for each treatment. The MIC and BSA were then log transformed to compensate for the dilution factor and a regression was conducted to ascertain the strength of the relationship between BSA concentration and silver nanoparticle toxicity. A regression was also conducted for ciprofloxacin as a comparison.

Results

The presence of relatively small amounts of BSA decreased the toxicity of silver nanoparticles by as much as 10 fold (Fig. 2). The MIC, with no BSA added, was approximately 63µg/L, which is significantly lower than the mean value (10.24mg/L) reported from other research into silver nanoparticles (Table 1). The log transformed regression showed a positive interaction of 0.3301 ± 0.0567 (R^2 = 0.8221, p<0.001 Fig. 3). In contrast, the interaction of the ciprofloxacin MIC and BSA concentration was 0.

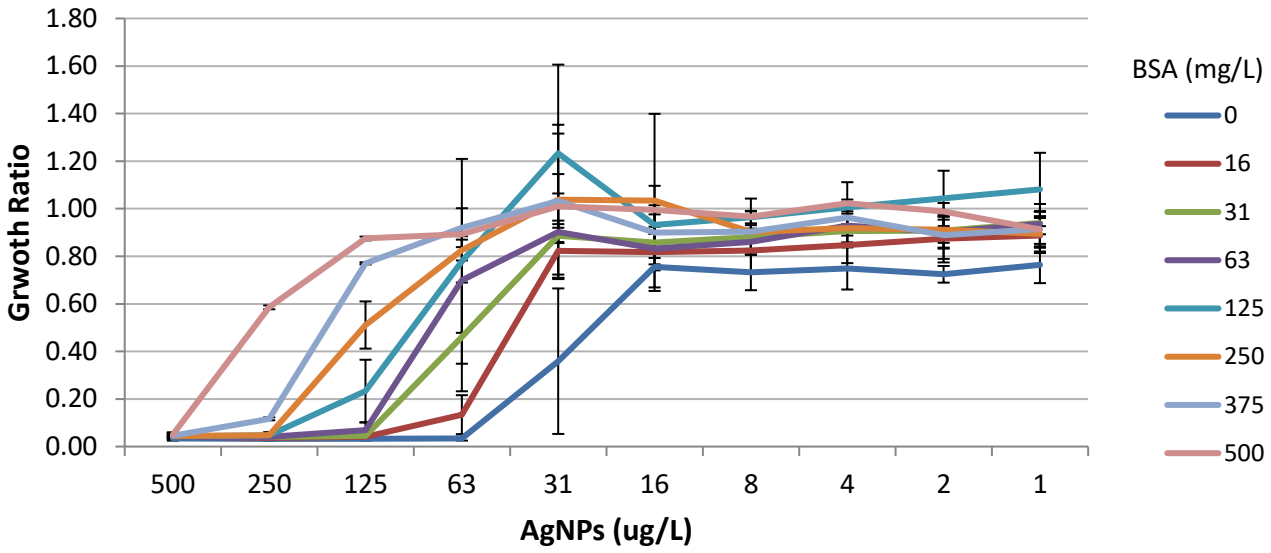

Figure 2: Average inhibition curves for each concentration of BSA. Growth was expressed as a ratio to a growth control, which contained no silver nanoparticles. Standard error bars are included at each silver nanoparticle (AgNP) concentration tested.

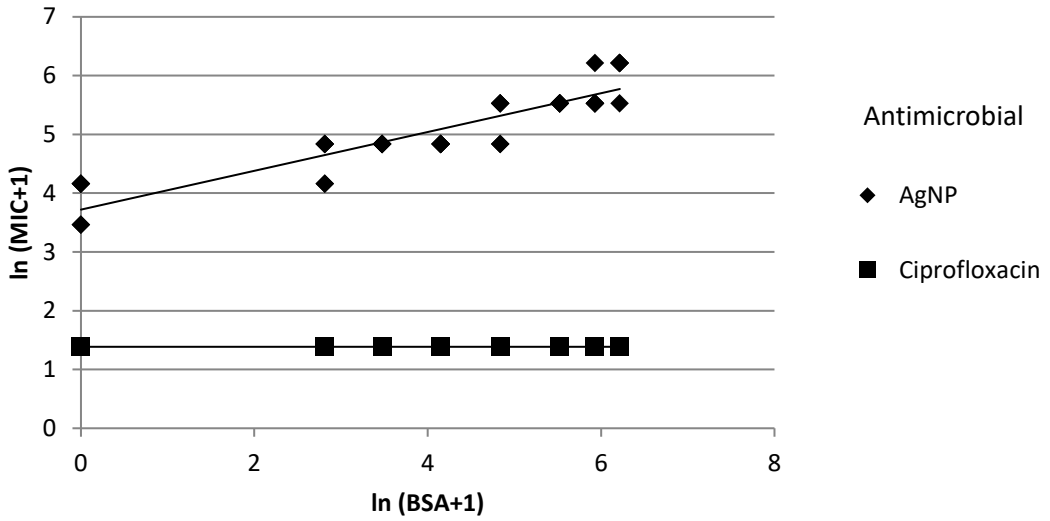

Figure 3: log transformed linear model of BSA against MIC for silver nanoparticles (AgNPs) and ciprofloxacin. The growth curves were log transformed to allow analysis via linear regression.

Discussion

M9 minimal broth contains only the minimal nutrients for bacteria to grow and has a protein concentration significantly lower than most commonly used broths, such as Lysogeny Broth, Mueller Hinton Broth, Nutrient Broth and Brain-Heart infusion broth. In the tests performed here, as BSA concentration was increased in M9 medium, so the effectiveness of the silver declined, and this was demonstrated in an increase in the MIC value (Figure 3). Because the silver nanoparticle MIC is strongly affected by protein concentration, proteins in media must interfere with the bioavailability of the nanoparticles. Consequently, while the publications listed in Table 1 can report on the relative efficacy of silver nanoparticles within studies, comparisons across studies are much more problematic, because of the potential influence of protein concentrations in each of the different media being used.

The interactions between protein and silver nanoparticles are caused by van der Waals and electrostatic forces (Liu *et al.* 2009).These forces cause the silver nanoparticles to agglomerate and colloidal stability is decreased. When silver nanoparticles agglomerate, the ionisation rate and bioavailability decrease and the toxicity of the silver nanoparticles decreases proportionately (Kittler *et al.* 2010).

Protein concentration, therefore, should be an important consideration when using silver nanoparticles as an antimicrobial. Hundreds of nanosilver products are commercially available for use in households (Anjum *et al.* 2013; Guo *et al.* 2015). These products will be applied in settings

that vary in protein concentration, and silver content should be adjusted accordingly. For example, nanosilver products involved in food preparation will be continuously exposed to high amounts of protein and will therefore need a higher concentration of silver to inhibit bacterial spread. If protein concentration is not considered, dosage or concentration of silver, may promote resistance at sub-inhibitory concentrations or cause needless pollution at high concentrations.

Interactions between silver nanoparticles and proteins may also affect the behaviour of silver in the aquatic environment. Unlike antibiotics, silver nanoparticles do not degrade in aquatic environments, but if they agglomerate around proteins and other organic matter, they may fall out of suspension. Studies into colloidal stability of silver nanoparticles in environmentally relevant conditions, have resulted in divergent conclusions. In pond water, Chinnapongse *et al.* (2011) found that the majority of silver nanoparticles were deposited in the sediment. In freshwater and seawater, Thio *et al.* (2011) found that there was minimal deposition of silver nanoparticles over a wide range of pH and ionic strength conditions. However, protein concentration was not considered in these deposition experiments. Decreased bioavailability of silver nanoparticles in aquatic settings could limit the chances of resistance developing via sub-inhibitory exposure. Even though most silver nanoparticles may settle into the sediment, there are multiple studies which describe the negative impact of silver nanoparticles on the ecology of aquatic systems (Gil-Allue *et al*. 2014).

There is little information available about the silver content of commercial nanosilver products. This raises concerns that the concentrations are not properly calibrated to usage. If silver nanoparticles are to be used in place of antibiotics and other biocides in industry, it is important to develop a standardised method to determine dosage so that silver nanoparticles are not misused.

The reported MICs for silver nanoparticles in the literature vary widely. It is now apparent that this could be simply due to the media in which the assays were conducted. When testing MICs for particular bacterial strains, it is important to consider the effect that the testing medium might have on the availability of silver. This suggests that uniform, low-protein media should be used in such tests. It also raises the possibility that silver nanoparticles may be less effective in biological systems where there is likely to be high protein availability. These considerations are important if the use of silver nanoparticles is to be properly managed in the post antibiotic era.

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Supplementary Information

Figure S1: TEM image of the 10nm PVP coated silver nanoparticles

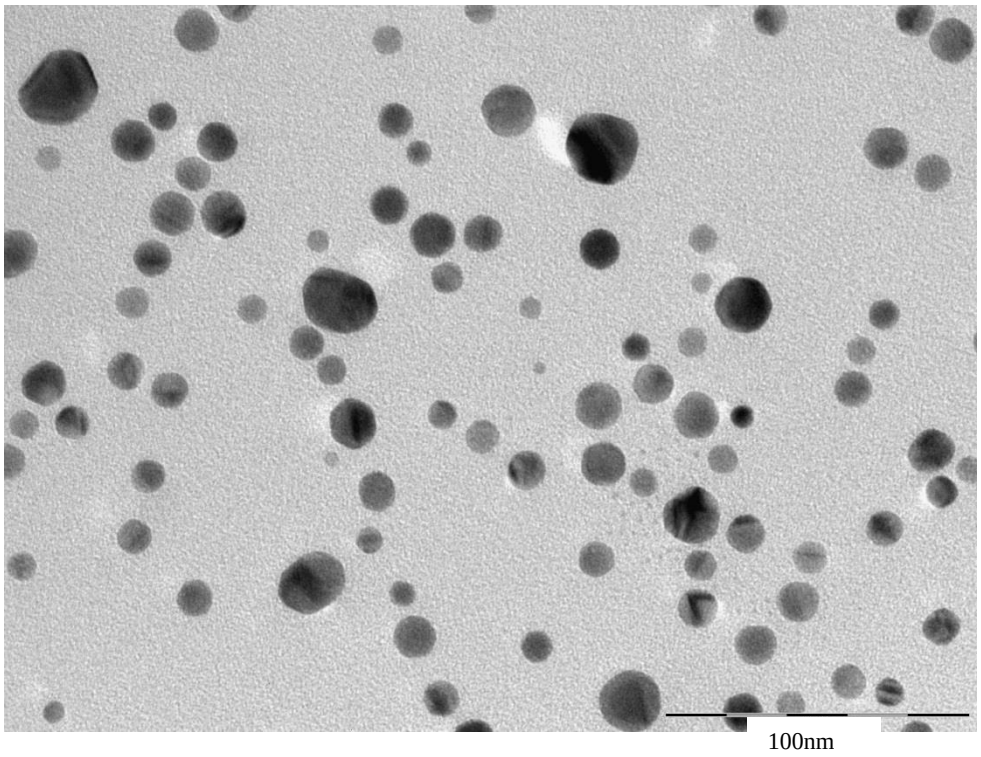

Figure S2: Vertical cross section of silver nanoparticles in LB agar shown under Olympus FLUOVIEW 1000 confocal laser scanning microscope. Silver nanoparticles fluoresce blue and red.

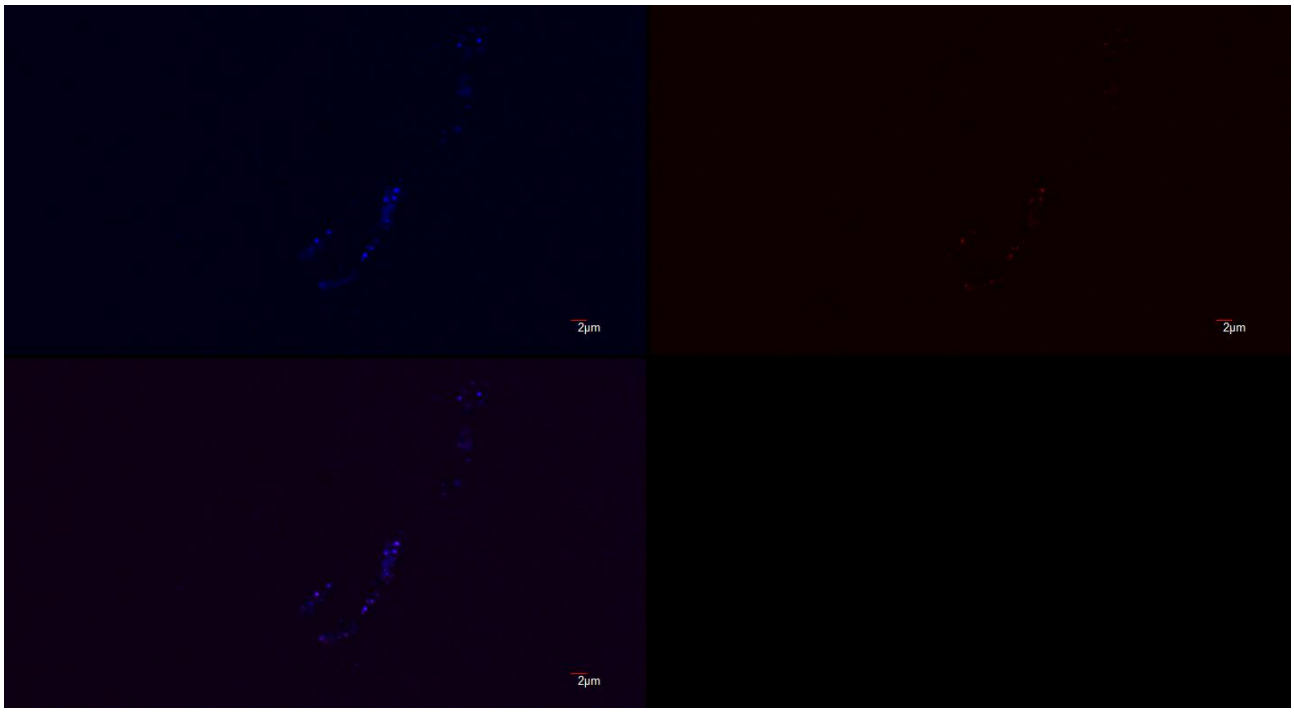

Figure S3: Lateral cross section of silver nanoparticles in LB agar shown under Olympus FLUOVIEW 1000 confocal laser scanning microscope. Silver nanoparticles fluoresce blue and red depending on the laser used to capture the image.

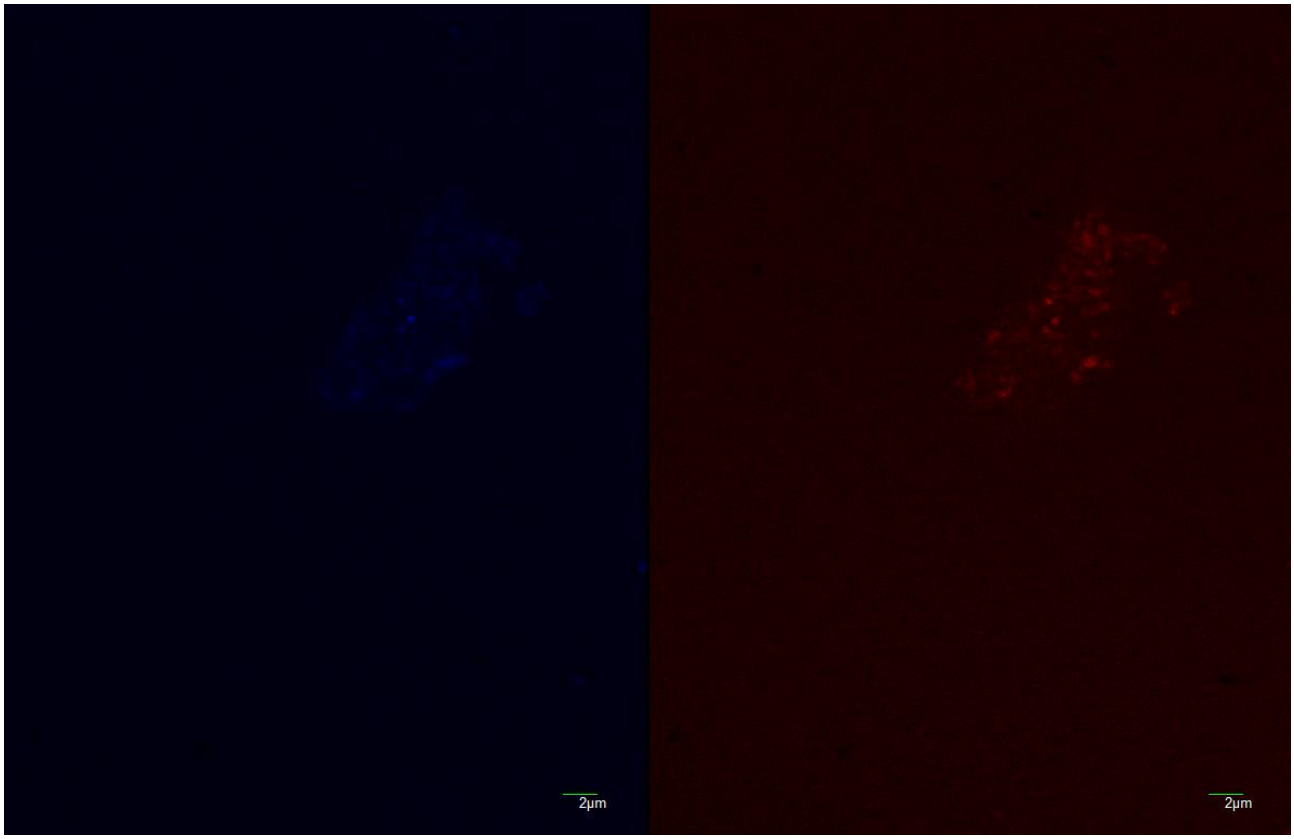

Figure S4: Magnification of silver nanoparticle agglomeration around an impurity in the media. Images were captured under a Olympus FLUOVIEW 1000 confocal laser scanning microscope. Silver nanoparticles fluoresce blue and red depending on the laser used to capture the image.

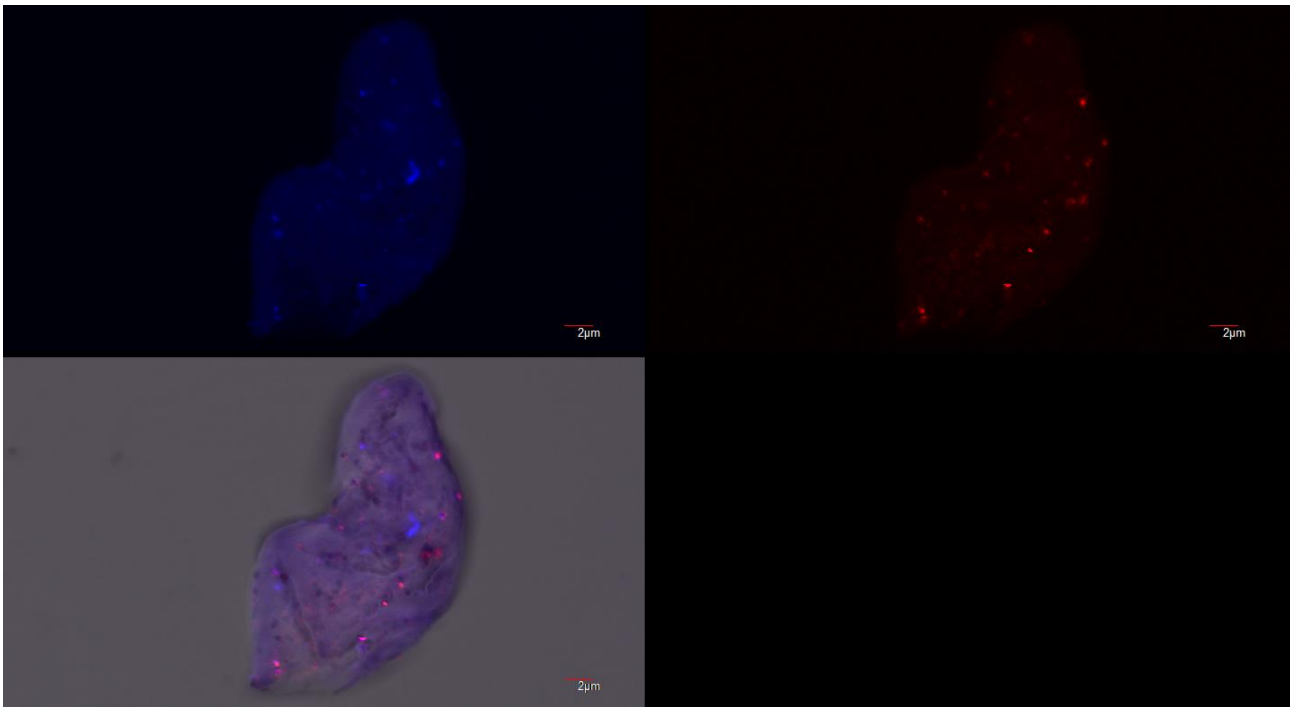

Figure S5: BOX PCR generated fingerprint of generation 5 of sub-inhibitory ciprofloxacin treatment.