Persistence of recombinant bacteria to antimicrobial silver

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Abstract

Silver, owing to its effective antimicrobial properties, has been used against a broad range of microorganisms. Silver is now utilized commonly in numerous consumer products, medical devices and clinical applications. However, the mechanism of action of the silver is not yet fully established and well-understood. In addition, it is also important to understand the biochemical and evolutionary pathways that give rise to resistance. Here, we report new genetic determinants for silver resistance in *E. coli* and explore aspects of their mechanism and laboratory evolution.

Initial exploration of the antimicrobial activity of silver showed that (1) antimicrobial ability of silver is time and dose-dependent; (2) Ag ions have much more antibiotic activity than silver nanoparticles (AgNPs) and (3) the antimicrobial ability of AgNPs is size-dependent. Further selection for resistance genes of *E. coli* using $AgNO_3$ and AgNPs led to the identification of several candidates, including *cysD* and *ycdB*, which displayed cross-resistance to Ag ion and AgNPs as well as Cu^{+} and Cd^{2+}. The genes *cysD* and *ycdB* conferred less resistance to metallic Ag(0) under anaerobic incubation than aerobic incubation. These results support that Ag^{+} ions are the main toxic agents of AgNPs. These novel anti-silver genes also endowed resistance to the antibiotics kanamycin and ampicillin; in these experiments, antibacterial synergy between kanamycin and silver, but not between ampicillin and silver, was also found. Quantification of oxygen radicals suggest that silver ion is bactericidal through production of reactive oxygen species and that silver-resistance genes prevent their generation.

The selected gene *ycdB* and control gene *cueO*, both of which led to increased silver resistance, encode Tat-dependent proteins, which are transported after folding from cytoplasm to periplasm. Chapter 2 focuses on several Tat-containing genes, which also gave more resistance to Ag ion. The 7 selected Tat sequence genes, including *torA, yedY, sufI, ycdO* and *hybA*, were recombinantly expressed in various truncated forms, showing that for *ycdB* and *yedY* deleting Tat sequences impaired export and silver-resistance ability, despite increased expression, but that for other Tat genes deleting Tat had little effect on either periplasmic translocation or resistance. In all cases, expression of the Tat export sequence alone or with the his-tag in absence of the gene led to suppression of resistance.

Finally, we explored the evolvability of selected genes, such as *yeaO, ydgT, iscA* and *ycdB* for silver-resistance. Evolved mutants of *yeaO* and *ydgT* were found that endowed increased resistance to silver compared to wildtypes. In these two cases, increased resistance to silver did not lead to increased antibiotic resistance. In short, several kinds of anti-silver genes were identified in our studies, showing various pathways rendering resistance to silver. Weak resistance functions for some genes were evolvable. Our studies provide a deeper insight into silver's mechanism of action and of the possible resistance pathways in bacteria, which may in some cases lead also to cross-resistance to antibiotics.

Contents

Chapter 1 Introduction

Silver, has been used effectively if unwittingly against a broad range of microorganisms since ancient times. Silver was arguably the most important antimicrobial compound until the introduction of antibiotics, after which silver and its compounds lay largely forgotten. However, due to the emergence of antibiotic-resistant pathogens, caused by their misuse, and with the steady development of nanoscale science, research on silver and its compounds has recently drawn more attention.[1] Silver nanoparticles, as well as various increasingly sophisticated silver-based compounds containing ionic silver (Ag^+) or metallic silver (Ag^0) exhibiting antimicrobial activity, are once more available for numerous applications.[2-5]

Compared to other metals and their compounds, such as platinum, gold, iron and nickel, silver has shown better bactericidal effects.[6] Many different assays *in vitro* and *in vivo* have explored the antimicrobial properties, biocompatibility and toxicity of silver, based on many different bacterial strains and cell lines.[7] Meanwhile, with the increased production volume of silver materials, a number of recent studies have focused on the environmental transformations of silver materials and their potential adverse effects on human health, because many products release silver in the form of nanoparticles, aggregates, or soluble ions during use, washing, abrasion, or disposal.[8-10]

Consequently, it is important to understand in detail how silver and its compounds exert their toxicity and to understand how bacteria may acquire silver resistance. Various mechanisms for the bactericidal activity of silver have been proposed and different preventive strategies have been suggested. The mechanism of silver toxicity in microorganisms has been attributed to multiple targets. Silver ions can react with electron-donor groups, such as nitrogen, oxygen, or sulfur atoms. These electron-donor groups are present in proteins, nucleic acids, DNA or cell membranes of bacteria.[11-14] Silver ions cause the release of potassium ions from bacteria.[15] In addition, one silver-resistance plasmid, pMG101, has been isolated from *Salmonella*, which contains genes that encode for a chemiosmotic ATPase efflux pump, and two periplasmic silver-binding proteins.[16] Although these mechanisms of anti-silver and binding between silver and proteins has been explored in specific cases, the mechanism of action of silver is not yet fully established nor well-understood.

The following sections will elucidate in detail on the use of silver as antibacterial agent, antibacterial activity of silver, human and environment toxicity of silver and silver resistance mechanisms.

1. The use of silver as antibacterial agent

Silver products, as microcidal agents, are common in medical and commercial applications. In hospitals, silver products have been extensively used in wards treating burns, where silver sulfadiazine and silver impregnated nylon cloth are used as antiseptics.[12,17] However, it appears that the use of silver preparations in burn treatment is rooted in tradition, whereas its actual effectiveness has also been questioned and widely criticized.[18] A topical ointment that contains 1% silver sulfadiazine is widely used in order to prevent and treat infections resulting from second or third degree burns, marketed as Silvazine or as Flamazine in USA and other countries.[19,20] Silver

sulfadiazine was initially used in creams, but recently this compound has also been incorporated directly into bandages used on burned skin surfaces; silver sutures are often used in surgical incisions to prevent infections.[21] Cerium nitrate-silver sulphadiazine (Flammacerium) as a topical treatment for cutaneous burns is deemed to reduce the inflammatory response, is claimed to decrease bacterial colonization, and provides a firm eschar (scab) for easier wound management.[20] Use of silver sulfadiazine instead of $AgNO_3$ is due to sulfadiazine ligand keeping the Ag(I) in a stable form, which is less subject to blackening by reduction.[19] The silver ion (Ag^+) is bioactive and in sufficient concentration readily kills bacteria *in vitro* and *in vivo*.[22] $AgNO_3$ has been used effectively as a biocide on burns but with the undesirable side effect of turning the burned tissue black from reduced Ag(0). Many different applications of silver drugs are currently in clinical trials (Figure 1).

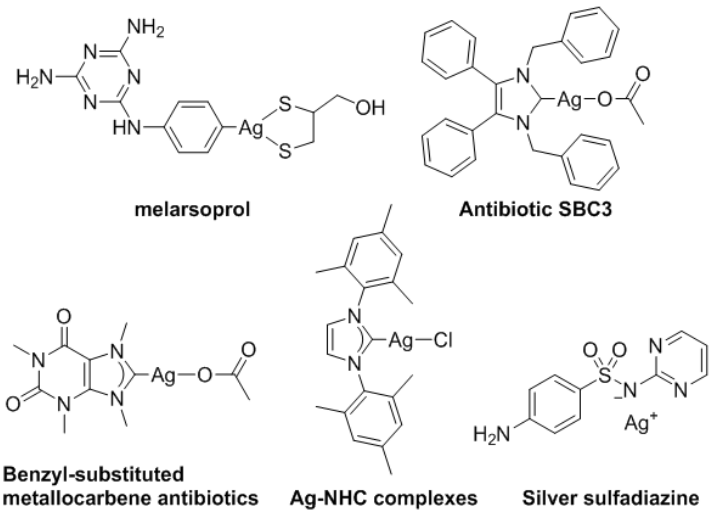

Figure 1. Antimicrobial and antiparasitic drugs approved and in clinical trials. Ref 19.

Silver-impregnated polymers of medical devices such as catheters and heart valves have been used widely to prevent the growth of bacterial biofilms.[23-25] Plastic in-dwelling catheters coated with silver compounds are used to retard the formation of microbial biofilms on the catheters and to prevent infection by nosocomial bacteria.[24] Clinical studies suggest that silver-coating urinary catheters and endotracheal breathing tubes may reduce the incidence of catheter-related urinary tract infections and ventilator-associated pneumonia, respectively.[25] Bacterial contamination is one of the most important complications of other medical implants, such as hip-replacements.[26] Bacteria on implants typically proliferate and cluster in multilayers of exopolysaccharides, known as biofilms, so bacteria can resist antimicrobial agents and immune responses.[27] In order to prevent infection on the surface of implant materials a coordination-polymer network based on silver, as a nanostructure coating, and an effective antimicrobial agent, has been synthesized and studied.[28]

Silver salts (such as silver nitrate) have also traditionally been administered to the eyes of newborn infants to prevent neonatal eye infections.[29] The use of silver for ophthalmologic treatment was widespread and recently extended. A colloidal form of silver was used successfully in the treatment of infected corneal ulcers, interstitial keratitis, blepharitis, and dacrocystitis. Amalgams, so-called 'silver fillings', containing about 35% Ag(0) and 50% Hg(0), is another traditional use of silver,

used to fill dental cavities, though this approach has been largely replaced by other materials due to the presence of toxic mercury in the amalgam.[11] The Ag^+-released slowly is thought to result in antibacterial activity. For example, Silver-zeolite, a hydrated aluminosilicate powder, is a new product of considerable interest, which can bind up to 40% of its weight as Ag^+ and incorporated into medical and dental objects.[30]

The antimicrobial properties of silver make it useful not only in biomedical settings but also in other commercial products. Since silver-impregnated bandages are available for infection prevention, it was a short step to embedding Ag in fabrics, including sleeping bags and sports socks.[31,32] This use has been proposed as a means of retarding microbial growth for hygiene and eliminating odor from sweat. Supermarket surfaces used for meat storage and display are sometimes 'silverized', again as a possibly useful biocide.[11] Metallic silver-copper-containing ceramic disks ('Clean Power Plus') are marketed as an alternative for users who might be allergic to laundry detergents.[11]

Silver-containing products are still used in water purification systems to control infectious agents (for example, *Legionella*), such as swimming pool water, hospital hot water systems and potable water systems.[33] The Russian MIR space station and the NASA space shuttle used silver to sterilize recycled drinking water.[34] Supermarket home-water purification units in the USA contain silverized activated carbon filters and ion-exchange resins (Brita Company).[34] Silver-copper ions can even replace corrosive chlorine to sanitize pools and tanks.[35] With the development of nanotechnology, AgNPs have gained extensive application as an antimicrobial agent in cosmetics[36] and the food industry, as well as for coating home appliances.[34] For example, the slow-release "nanosilver" linings of refrigerators, mobile phones, clothes, plasters, and toothbrushes are also marketed and advertised.[34] Silver, as one of many options, can replace toxic chromated copper arsenate to be used as a wood preservative. Nanosilver inks and coatings on paper tout their ability as antimicrobial agent used in inhibiting the spread of bacterial infection. Silver-based ionic liquids can be used to clean up petroleum waste products. Silver has even been used to plate instruments, such as flutes.[34]

Table 1. Noble metal concentration in some cosmetics, determined by atomic absorption spectroscopy after chemical pulping. The content of platinum was below the detection limit of 15 ppm

Sample	Noble metal concentration [ppm]	Packaging size [mL]	Noble metal per package [mg]
Silver toothpaste	0.1	75	0.0075
Silver shower gel	2.7	200	0.54
Silver hand cream	2700	75	202.5
Silver deodorant (roller)	950	50 mL	47.5 mg
Gold night cream	2.4	50 mL	0.12 mg
Platinum anti-wrinkle cream	< 15	50 mL	< 0.75 mg

The noble metal contents in some cosmetics have been measured and summarized by atomic absorption spectroscopy (AAS; Table 1).[33] The concentrations of silver were found to cover a range

of more than three orders of magnitude. The potential uses of Ag materials is very extensive, and this is only a partial list for silver used as the antimicrobial agent.[34] In summary, use of silver is traditionally routed but is being applied increasingly to many new uses.

2. Antibacterial activity of silver by binding to biomolecules

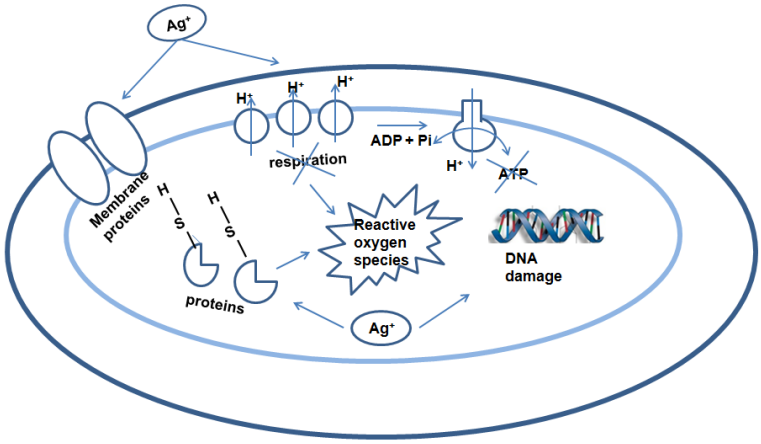

Figure 2. Antimicrobial effects of Ag^+. Interaction with membrane proteins and blocking respiration and electron transfer; inside the cell, Ag^+ ions interact with DNA, proteins and induce ROS production.

The antimicrobial activity of silver compounds has been studied for a long time.[34,35] Silver cation is a soft Lewis acid, which has an affinity to sulfur but also to nitrogen. So there are many possibilities to disturb biochemical processes, which make the mechanism of action of silver ion often inconclusive (Figure 2). Silver ion can rapidly interact with thiol groups of membrane proteins of microorganisms, leading to the formation of an S–Ag coordination bond. This interaction has been suggested to prevent respiration and electron transfer, which in turn hampers the induction of successful rescue mechanisms.[36-38] Blocking respiration and electron transfer causes a collapse of the proton motive force, which results in de-energizing of the membrane, ultimately leading to cell death.[37-39] When Ag^+ ions enter the cytoplasm, they are also able to bind to the guanine base (N7 atom) and interfere with DNA replication.[4] Meanwhile, silver ions can interact with other donor atoms, which are present as amino, imidazole, phosphate, carboxyl, or thiol groups in DNA or proteins.[5] Coordination of Ag ions can lead to conformational changes and to inactivation of enzymatic functions. The activity of silver ions against some bacteria can be neutralized by cysteine and other thiol compounds.[40] However on the basis of a 1:1 stoichiometry the three basic amino acids arginine, lysine, and histidine are the strongest silver ion binders (Table 2).[41] In general, coordination sites present in an amino acid can be classified as three types: (i) the amino nitrogen-donor at the N-terminus, (ii) the oxygen atoms of the carboxylic group of the C-terminus, and maybe (iii) the heteroatom-containing side chains in various possible coordination modes (Figure 3).[42] For AgNPs, a prime pathway realizing its toxic biological activity appears to be the release of Ag ion, a view which is now increasingly accepted. These ion-based toxicity pathways of Ag particles have been proposed to function by (i) generating a continuous flux of Ag^+ bound on substrates or embedded in matrices or (ii) transporting active Ag^+ to susceptive biological targets on cell membranes or within cells ensuing particle attachment or endocytosis.[43,44] The toxic effect of AgNPs against a broad

spectrum of bacteria and viruses has been reported.[45-52] Several studies suggest that the toxicity of AgNPs depends on their size, being responsible for their specific physiochemical characteristics. The smaller the nanoparticles are, the larger the surface available for interaction is, resulting in a higher specific activity.[53,54] However, the mechanism of how AgNPs exert their toxicity is not yet completely known.

Table 2. Calculated EnthalpiesΔH° and Free Energies ΔG° (kJ mol^{-1}) for the Amino Acid −Ag(I) Complexes

α-amino acid	ΔH^{oa}	ΔG^{oa}	*α-amino acid*	ΔH^{oa}	ΔG^{oa}
glycine (Gly)	206.1	170.1	glutamic acid (Glu)	239.9	203.1
alanine (Ala)	212.8	176.8	tyrosine (Tyr)	239.9	202.3
valine (Val)	216.1	181.0	asparagine (Asn)	250.8	217.4
leucine (Leu)	219.5	185.6	tryptophan (Trp)	260.0	221.5
isoleucine (Ile)	221.1	188.9	methionine (Met)	262.1	219.9
serine (Ser)	224.5	190.2	glutamine (Gln)	264.2	225.5
cysteine (Cys)	230.2	194.4	histidine (His)	284.2	249.1
threonine (Thr)	233.2	199.8	lysine (Lys)	296.8	260.8
aspartic acid (Asp)	232.4	199.0	arginine (Arg)	336.5	279.8
proline (Pro)	234.5	199.8	phenylalanine (Phe)	236.2	198.6

[a]Estimated from ref 41.

3. Antibacterial activity of silver mediated through oxygen radicals

All aerobic organisms produce a by-product of aerobic respiration, reactive oxygen species (ROS), such as singlet oxygen, hydrogen peroxide, superoxide radical anion, and hydroxyl radical (equation 1).[41] ROS are short-lived reactive oxidants which are highly toxic in that they cause damage to biomolecules, such as proteins, DNA, RNA and lipids, so protective mechanisms have evolved by all organisms.[55] This protection in bacteria is reflected by the presence of two sensor-regulator proteins called SoxR and OxyR. SoxR-mediated induction of transcription upon silver treatment is similar to that found upon paraquat-treatment, a known superoxide-radical generator.[56] Superoxide anions can give rise to release of iron from iron-sulfur clusters of the respiratory chain enzymes, which in turn can induce the generation of hydroxyl radicals by the Fenton reaction.[57] Ag^{+}-treated *E.coli* cells exhibited detectable increases in hydroxyl radicals compared to untreated cells.[26] It was proposed that Ag binding to the thiol of anti-oxidative enzymes block their activity, preventing detoxification of the generated ROS. Furthermore, bacteria grown anaerobically are often less sensitive to Ag^{+} ions, which putatively reflects the influence of ROS production on the antibacterial activity of Ag^{+}.[56,58] However, the formation of ROS has been reported to have only a negligible contribution to the antimicrobial properties of silver compounds in some reports, indicating that other factors are at play.[26] The contribution of ROS formation is discussed controversially not only for Ag(I) but also for AgNPs. Nanosilver particles have been reported to produce lipid-oxidizing peroxide intermediates after particle attachment to cell membranes during reactive dissolution.

However, the mechanism of antimicrobial action of AgNPs themselves, the so-called particle-based mechanism, remains controversial.[59,60]

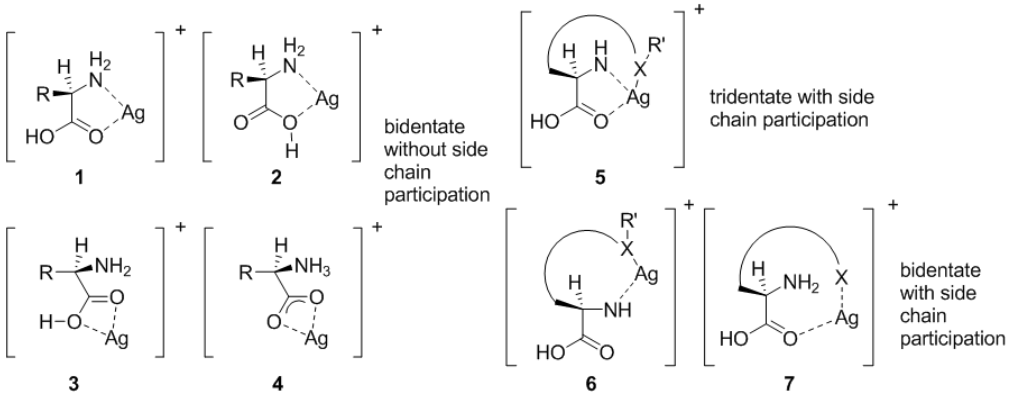

Figure 3. Selection of possible coordination modes between Ag(I) and amino acids. R represents the side chain of the amino acid in general, if it does not participate in the silver binding, while X represents the coordinating heteroatom present in the side chain. ref 41

Equation 1: $O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^-+2H^+} H_2O_2 \xrightarrow{e^-} OH^- + OH\bullet \xrightarrow{e^-+2H^+} 2H_2O$

4. Human and environment toxicity of silver compounds

Speciation of silver-The speciation and bioavailability of silver are important for understanding its potential risk. Different forms of silver have different degrees of toxicity. Silver, which is soluble in water and unattached to any other atoms while in solution, is referred to as "free silver". In general, the free silver is the most toxic form. Some silver compounds such as silver sulfide and silver thiosulfate discharge ionic silver very slowly because of very low solubility or complexation of the silver (K_{SP} (25 °C): $Ag_2S = 6 \times 10^{-51}$ mol^3 L^{-3}, $AgCl = 1.8 \times 10^{-10}$ mol^2 L^{-2}, $Ag_3PO_4 = 8.89 \times 10^{-17}$ mol^4 L^{-4}). These compounds are much less toxic than silver nitrate to aquatic organisms. Because of its tendency to form almost insoluble compounds in natural waters and sediments, it has been argued that it is in practise impossible for bioavailable silver ions to reach sufficiently high concentrations to cause toxicity in marine environments. So the chance for organisms to be affected in the long-term appears to be minimal.[61] However, it is crucial to precisely measure silver in the environment and to determine the form of silver to predict the potential for any adverse effects.

Toxicity of silver compounds-There has been a sustained battle for more than 100 years between supporters of uses of silver-preparations for health and medical benefits and government agencies regulating claims and products.[62-64] However, with the tremendous boost of silver products in wound care products, medical devices, textiles, cosmetics and domestic appliances, the issues of safety and potential risks for the human body and environment have also become increasingly urgent and relevant.

Silver contact with the human body is mainly through three ways: respiration, inhalation and skin absorption. Compared to most heavy metals, silver is better tolerated by the body and it is relatively nontoxic to mammalian cells. The amount of silver contacted by most people on a routine bases is

very low, primarily from food and drinking water, but also to a smaller extent from air (1×10^{-6} $\mu g L^{-1}$).[65] In the literature, only few studies describe *in vivo* results concerning the cytotoxicity of silver.[66] The most common observable changes upon acute exposure are argyria and argyrosis which are related to prolonged exposure to silver compounds, characterized by an irreversible deposition of silver selenide and silver sulfide precipitates in the skin and the eyes, respectively. For example, a clinical case of argyria was reported following months of eating a silver-containing food supplement.[67] Another curious case relates to systemic argyria in a patient who chewed photographic films in the long term after he gave up smoking.[68] These cases display that the gastrointestinal tract is a place where the sparingly soluble silver halides from the photographic film were easily mobilized. Silver salt can be reduced within the tissues. This reduction is photoactivated and causes the skin to function in a manner similar to photographic film. The year-long application of a nose spray containing protein-bound silver also resulted in argyria.[69] The affected area became bluish-gray and got worse in the presence of sunlight; however argyria and argyrosis are not life-threatening and are not related to irreversible tissue damage.[70-72]

In some reports a chronic inflammatory reaction occurred when patients were treated with a silver-coated heart-valve even though the silver blood concentration did not exceed 22 ppb (22 $\mu g\ L^{-1}$);[73] other studies described a simple way toward hybrid fluorescent microgels *via* photoactivated synthesis of Ag nano-clusters in the microgel hosts, with good biocompatibility and no cytotoxic effects.[74] It is well-known that silver toxicity is a dose-dependent process, so these contradictory results can be attributed to the variation of the silver concentration acting on different cell types.[21,41] After an overexposure to silver, silver might be absorbed and transported by the bloodstream, accumulating in organs and tissues such as liver, skin, kidney, spleen, heart, lung, olfactory bulb, corneas, gingival mucous membranes, brain, and testes.[75-77] Several systemic clinical side effects of silver may emerge, such as leucopenia, liver and kidney damage. Reports on the toxic effects of silver for nerve tissue, centrally and in the periphery, are rather rare. However, silver has been claimed to cause brain damage,[78] seizure,[79] and even a persistent vegetative state.[80] But since the severity of tissue damage is dose dependent, low concentration of silver compounds appears to be administrable without lethal side effects. Toxic effects of AgNPs have been studied for different *in vitro* and *in vivo* mammalian systems.[81-83] Indeed, AgNPs can potentially cause toxic effects at the tissue and cellular level, such as inflammation, immune-cell activation, depletion of glutathione (GSH) level in association with mitochondrial dysfunction.[84] Arora *et al*. confirmed *in vitro* clear signs of oxidative stress and increased lipid peroxidation when human cell lines were exposed to AgNPs.[85] Finally, all of these processes gradually lead to apoptosis.[86] How exactly AgNPs induce cytotoxicity is still not well understood. Moreover, at the level of the whole organism additional complexity arises: for example smaller particles not only have a higher toxicity compared to larger particles, but they can also pass more easily through biological barriers, such as the blood−brain or the blood−testes barrier.[87]

Maximum concentrations of total silver that have been recorded in selected non-biological materials is 2.0 $\mu g/m^3$ in atmospheric dust; 6.0 µg/litre in groundwater near a hazardous waste site; 300 µg/litre in treated photoprocessing wastewaters; 43 mg/litre in water from certain hot springs; as much as 100 mg/kg in crude oils; and 150 mg/kg in some sediments.[2] It is important to mention that only a small part of the total silver in each of these compartments is biologically available. The effect of silver on the environment has been discussed in some review articles, but sometimes

distinctly different conclusions were reached.[88-92] The opinions range from "problematic" to "no problem at all". Although silver enters waste-water from a variety of sources, both industrial (e.g. photographic and electronic industries) and from consumer products, a recent assessment came to the conclusion that currently biocidal uses of silver (including silver nanoparticles, as well as silver in other forms, such as ionic silver) make up not more than 15% of the total silver flow into wastewater.[93] In a general way, silver ion possesses less toxicity to freshwater aquatic organisms under conditions of low dissolved silver ion concentration and increasing water pH and hardness.[94]

5. Silver resistance mechanisms

With the increase in occurrence and number of antibiotic-resistant strains, silver and its compounds have been reused as antimicrobial agents after being largely abandoned for about 50 years in response to the discovery and development of antibiotics. As an antimicrobial agent, the biggest advantage of silver is its presumed multitarget mechanism, so development of resistance in the cell is in theory very difficult and is thought to require plenty of mutations.[95] However, some bacterial strains regularly exposed to relatively high concentrations of silver have been discovered that are able to grow in this silver-rich environment.[96-99] The discovery of these silver-resistant strains caused an intensified interest in the machinery of silver resistance. Despite several reports on silver resistance in bacteria and the description of several silver-resistant plasmids,[16,100-102] the molecular mechanisms behind the silver resistance remains incompletely understood and two leading theories are currently debated: The first one is the accumulation and storage of silver, involving reduction of toxic silver ion to less harmful Ag(0),[103] and the second one is a silver efflux mechanism, silver ion being transported out of the bacteria.[104] These two theories are both based on the concept of detoxification of the cell.[105] The anti-silver plasmid pMG101 is the most extensively studied and most frequently cited model.[16,106]

The first proposed mechanism, accumulation and storage-based mechanism, is largely based on the silver resistant *P. stutzeri* strain AG259, which was discovered from silver mines and analyzed by Haefeli *et al.* in 1984.[106] This kind of strain had the unique capability to reduce Ag^+ ions into Ag nanoparticles and accumulate them within its cell. Ionic silver (Ag^+) is well-known to be toxic to bacteria, so this strain minimizes the Ag ion toxicity by reduction to metallic silver. Notably, in the particular context of Ag nanoparticles bio-synthesis by bacteria also opened up new exciting avenues for eco-friendly, large-scale, and economically viable shape-controlled synthesis of nanomaterials.[107] The pKK1 plasmid was confirmed to encode for Ag-resistance in *P. stutzeri* strain AG259.[100] The Ag-resistant (AG259) and the Ag-sensitive (JM303) *P. stutzeri* strains were both able to accumulate silver, but TEM and energy dispersive X-ray analysis showed that only the resistant strain was able to form dense metal deposits.[108,109] This is possibly because of high sulfur content which was found in the resistant strain AG259. The production of hydrogen sulfide was deemed to play a role in the formation of metal deposits. The bio-synthesis of nanosized crystals of diverse types and shapes were also reported by using the same silver-resistant strain, indicating that small changes in the experimental conditions might have a powerful impact on the outcome of the experiment.[107] *Acinetobacter baumannii* BL88 was also found to accumulate and retain silver. Its plasmid pUIP199 was discussed to be responsible for the accumulation of silver ions and that silver resistance from the plasmid was transferable from *Acinetobacter baumannii* to *E. coli*. However, the exact location of accumulation of silver, either on the surface of the cell or in its interior, was unclear.[101]

The second mechanism of silver resistance, first postulated by Silver *et al.* in 1999, involves the existence of efflux systems. Plasmid pMG101 is a 180-kb IncH1 silver resistance plasmid[120] that also endows resistance to several antibiotics and other heavy metals, such as mercury and tellurite.[121] Research into this plasmid revealed that the silver resistance is encoded by the *sil* gene cluster (Figure 4). This resistance system consists of two Ag(I)-binding proteins (SilE and SilF) and two different efflux pumps, a P-type ATPase (SilP) and a membrane potential-dependent three-polypeptide cation/proton antiporter (SilCBA) (Figure 6). These sil-proteins are only expressed when cells are grown in the presence of silver, and their expression is regulated by a system consisting of a membrane kinase sensor SilS and a transcriptional regulatory responder, SilR.

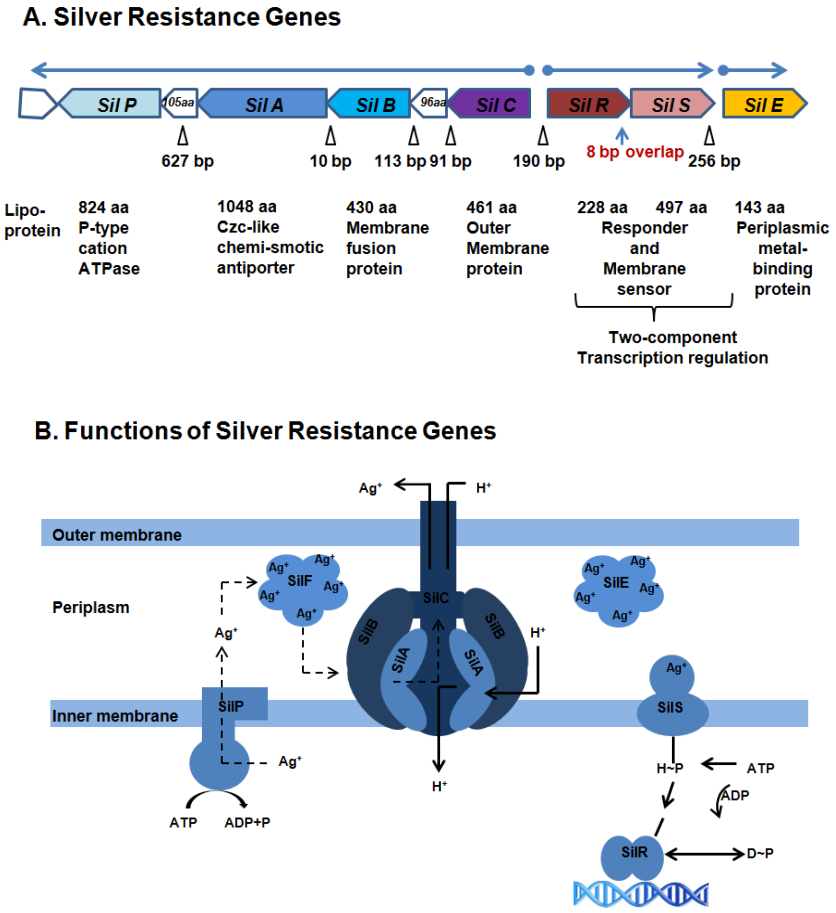

Figure 4. Silver resistance genes, transcripts and protein products. A: Top line shows the mRNAs. The open boxes indicate di¡erent genes or ORFs and their orientations. Nucleotides (nt) between genes and the sizes of gene products in amino acids (aa) are marked. B: The proposed function of each gene product, deduced from homologies to known proteins (from [17])

SilE and SilF are both thought to bind silver, but they differ in their composition and mode of action.[122] SilE is a small periplasmic Ag(I)-binding protein and combines to Ag(I) ions specifically at the cell surface, rendering the first line of resistance against Ag(I) toxicity. SilE contains 143 amino acids and is 47% identical to the metal binding peptide PcoE from the plasmid copper resistance system of *E. coli*.[16] SilE has a higher specificity for Ag(I) than Cu(II) and Cd(II).[17] In SilE, 10 histidine residues are located as the primary silver binding sites. Binding of Ag(I) to the SilE

protein is considered to result in a structural change in protein folding from a random coil to a predominantly α-helical system. Compared to other metal-binding proteins such as metallothionein, SilE has no cysteine residues (Figure 5).

The second silver binding protein SilF encoded in the sil system is a homologue of the chromosomally-located sequences CusF in *E. coli* (50% identity). CusF is part of the Cus efflux system that gene cluster is mainly involved in copper resistance, and it was proved to also confer a certain degree of silver resistance via a "methionine shuttle". The small periplasmic protein CusF is proposed to act as a metallochaperone. In contrast to other known metal carrier proteins, CusF functions as a monomer in which three amino acids (Met47, Met49, His36) fully coordinate Ag(I), and Trp44 caps the metal binding site of the molecule.[123,124] CusF possesses high binding affinity for Ag(I) over for Cu(I). This may be attributed to the different affinities of those ions to sulfur ligands or to their different sizes. In the sequence of SilF the four amino acids forming the principal binding motif are conserved. Consequently, SilF is predicted to bind the silver cation by a single histidine and two methionine residues from a β -sheet structure, similar to CusF. In the current models, both metal binding proteins CusF and SilF are in charge of the capture and subsequent transport of Ag(I) to the corresponding efflux pumps, where the toxic metal ion is finally eliminated from the cell.

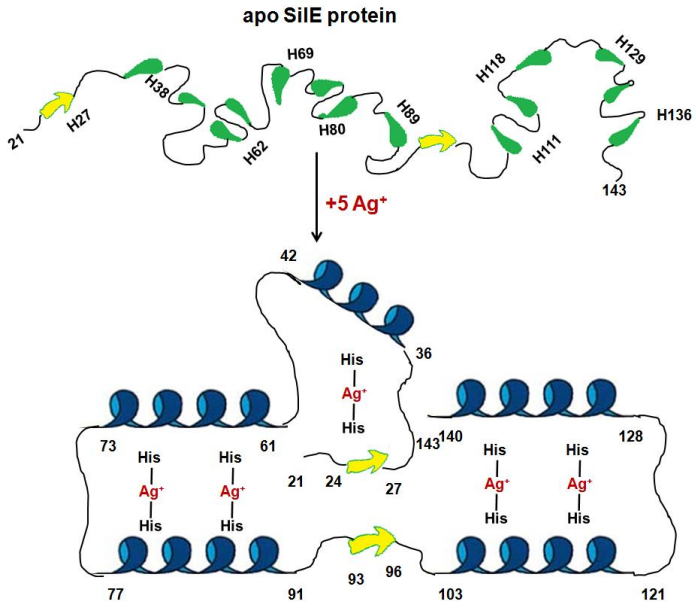

Figure 5. Model for Ag(I) binding and folding of the periplasmic Ag(I)-binding protein SilE. Top: 122-amino acid processed SilE protein after removal of 20-amino acid leader sequence with positions of the 10 histidine residues noted. Bottom: secondary structure predictions of K-helical (coils) and L-sheet (arrows) regions from standard software and predicted cross-linking of five Ag(I) cations by 10 histidines (from[17]).

The silver resistance determinant, SilP (Figure 4A , left), is recognized to be a member of the heavy-metal resistance efflux P-type ATPase family ATPase that probably pumps Ag(I) from the cell cytoplasm to the periplasmic space (Figure 4B).[125] SilP includes all the specific features of a P-type ATPase,[126] but there is one salient difference between SilP and other heavy-metal efflux ATPases, which is that ATPases have one to six copies of a Gly-Met-X-Cys-X-X-Cys sequence towards the N

terminus, whereas SilP lacks cysteines in a comparable location. Instead of a cys-rich region, SilP has His_5-Asp-$His_{2motifs}$. There is no silP homolog in the region of the *E. coli* chromosome corresponding to silABCRS homologs. SilCBA consist of a three-polypeptide membrane potential-driven cation/proton exchange complex (Figure 4) that is a member of the "resistance, nodulation, and cell division" (RND) superfamily of cation efflux pumps.[16] The components of this presumed Ag(I) efflux system include (a) inner membrane SilA, the large 1048-amino acid proton/cation antiporter, (b) the outer membrane protein SilC, which assures transport across the periplasmic space of Gram-negative bacteria and directly to the outside of the cell without release into the periplasmic space and (c) a paralogous class of 'membrane fusion proteins' SilB, which anchors into the inner membrane and connect to the outer membrane protein, SilC.

Although silver resistance and its regulation has been studied in molecular detail in this Sil system, there are still discussions whether there is a real resistance towards silver or whether resistant microbes only eliminate the silver species.

Here, we describe how overexpression of several different genes in *E.coli* can provide not only independently increased resistance to antimicrobial silver, but also to other metals[127] and antibiotics. Our data support the notion that silver nanoparticles achieve their antimicrobial activity chiefly by releasing Ag ion and that killing is mediated by reactive oxygen radicals. Further, we identify several mechanisms by which bacteria overcome such metal-ion mediated antimicrobial action and describe how low-level resistance may evolve toward increased silver resistance.

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Chapter 2 Selection of genes conferring silver resistance in bacteria

Introduction

After their discovery, antibiotics largely replaced silver and other antimicrobial compounds, which became nearly forgotten for almost 50 years. However, the increase in antibiotic-resistant bacteria has revived the interest in the antimicrobial effects of silver and its compounds. Silver, as a broad-spectrum antimicrobial agent is increasingly used in medical and consumer products. However, exactly how silver exerts its the antibacterial action remains largely unclear. In addition, the question whether pathogenic bacterial strains may eventually evolve resistance against silver is a little-explored question of great practical relevance. Moreover, it is also of concern that silver may provide an indirect selective pressure for antibiotic resistance in bacteria,[1,2] further underlying the importance of understanding resistance mechanisms in bacteria, not only to antibiotics, but also to other antimicrobial compounds such as silver.

A declining pipeline of clinically useful antibiotics has made it imperative to develop more effective antimicrobial therapies, particularly against difficult-to-treat Gram-negative pathogens. Numerous studies have attempted to resolve this question.[3,4] As a result, increasingly more pressure has been put on developing new antibiotics or searching some new approaches for overcoming the bacterial resistance, like altering the structure of antibiotics or using dual action antibiotics. For example if bacteria have resistance to one of antimicrobial agents, another may be used to counteract resistance or kill in a complementary way, such as clavulanic acid has been used to incapacitate the β-lactamases, to support the action of β-lactam antibiotics.[5] Therefore, research into resistance mechanisms and novel antimicrobials are complementary.

Amongst all inorganic antimicrobial agents, silver elements and compounds of nanoparticles have been the most extensively tested ones as antimicrobials. It is possible that the inherent antimicrobial activity of silver in combination with conventional antibiotics can improve their efficacy.[6] In summary, the exploration of the mechanism of silver antimicrobial action and mechanisms of resistance are relevant in light of recent efforts in combining the potential therapeutic effects of antibiotics and inorganic nanosilver or silver ion, in the search toward new and improved antibiotics.[3]

To research bacterial resistance to silver and to find methods to improve the antimicrobial activity of silver, it is useful to assess the susceptibility of a bacterial strain to the antimicrobial agent, as defined by the Minimum Inhibitory Concentrations (MIC). The MIC is defined as the lowest concentration of an antimicrobial that will completely inhibit the visible growth of a microorganism after certain incubation time. The measure of MIC is important to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents.[7] For example, one can determine that a given plasmid gives more resistance to silver by comparing MIC value to that of a control strain. The higher the MIC value is, the more resistance the clone is to the antibacterial agent tested.

To identify genes conferring resistance to antimicrobial silver, either in the form of silver nitrate or citrate-stabilised silver nanoparticles, we exploited *E.coli* as a model system, selecting plasmids from the ASKA library that raised the minimal inhibitory concentration for these inorganic antimicrobial compounds. The ASKA library contains more than 4000 *Escherichia coli* K-12 Open Reading Frames, representing the majority of this model bacterial genome.[8] Following selection and validation of several silver-resistance genes, the effects of induced overexpression with IPTG (isopropyl-β-d-thiogalactoside); the effects of oxygen; as well as cross-resistance with different silver compounds and other antimicrobial compounds, including antibiotics were explored in further detail. Here, plasmids-encoded genes conferring increased resistance to silver were obtained and validated against the control strain (strain carrying plasmid without any insert). To research the effect of cross-resistance, different types of silver preparations and other metal ions were tested. In particular, we compared the toxicity of silver nitrate to metalic silver(0) stabilized in citric acid or gelatine (a generous gift by Nanotrade, Czech Republic) and by conducting antibacterial assays under both aerobic and anaerobic conditions. Whereas zero-valent silver metal is largely insoluble in water,[9] Ag^+ can be released from AgNPs following oxidation of Ag(0) on the nanoparticle surface.[10] The resistance of anti-silver genes to antibiotics and synergistic of antibiotics in combination with silver were also tested. Finally, the production of reactive oxygen species (ROS) induced by silver ion was also examined.

2.1 Material and Methods

Materials

Escherichia coli XL1-Blue was purchased from Stratagene. Luria Broth (LB) medium was used as a rich medium. Tetracycline and chloramphenicol were used at final concentrations of 5 and 34 μg/ml, when added to either liquid or solid medium. Liquid cultures were aerated by shaking on a rotary shaker (200 rpm), and all growth incubations were at 37°C. Agar (used at 1.5%) and agarose (used at 1%) were purchased from Invitrogen. Electro competent cells and chemical competent cells of *E. coli* were prepared by standard protocols. IPTG was used at a final concentration of 1 mM. All other chemicals were purchased from Applichem, unless otherwise specified. The ASKA (A complete Set of Escherichia coli K-12 Open Reading Frame Achive) library comprises every *E. coli* open reading frame cloned into the expression vector pCA24N.[8] 10 and 50 nm AgNPs were purchased from Nanocomposix and stabilised with 2 mM citrate acid. These particles had a reported mean size of 10±1.8nm and 50±5.2nm. The AgNPs solution was diluted in Milli-Q water. Metallic silver(0) and ion silver colloid were provided by Nanotrade.cz. The Live/Dead dyes (SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI)) and 3′-(p-hydroxyphenyl) fluorescein (HPF) dyes were purchased from Life Technologies (Figure 1).

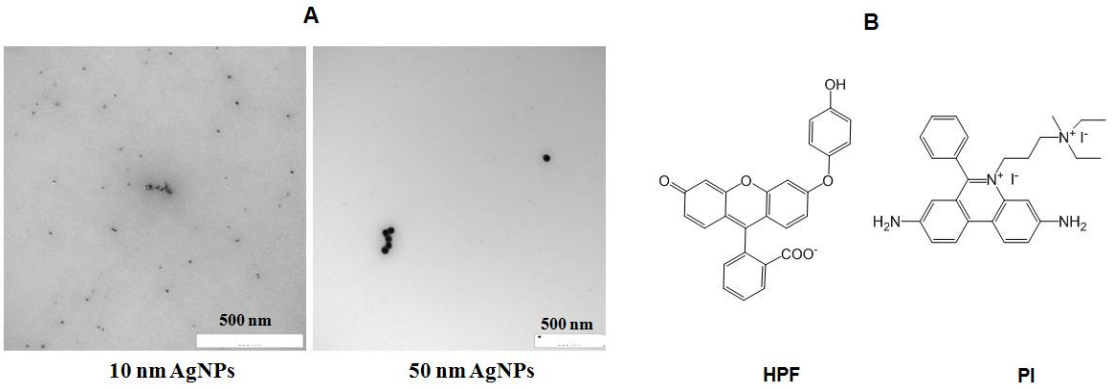


Figure 1. (A) Transmission electron microscopy images of 10 and 50 nm AgNPs. (B) Nonfluorescent hydroxyphenyl fluorescein (HPF) and propidium iodide (PI).

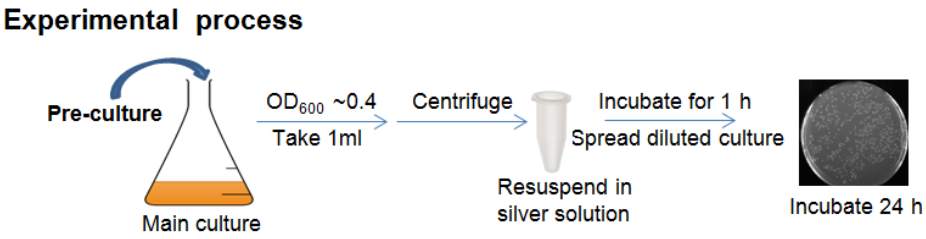
Experimental methods

2.1.1 Transmission Electron Microscopy (TEM)

The size and the morphology of the silver nanoparticles purchased from Nanocomposix were examined by transmission electron microscopy (TEM) using a Philips EM420 instrument at 120 kV, at the Biozentrum Microscopy Facility. TEM samples were prepared by dripping a drop of AgNPs solution on copper grids and placing the grids at room temperature to make solution evaporate (Figure 1).

2.1.2 Testing the effect of size and concentration of AgNPs and reaction time on antibacterial activity— *E. coli* XL1-Blue

Scheme 1. Schematic representation of experimental process of MIC



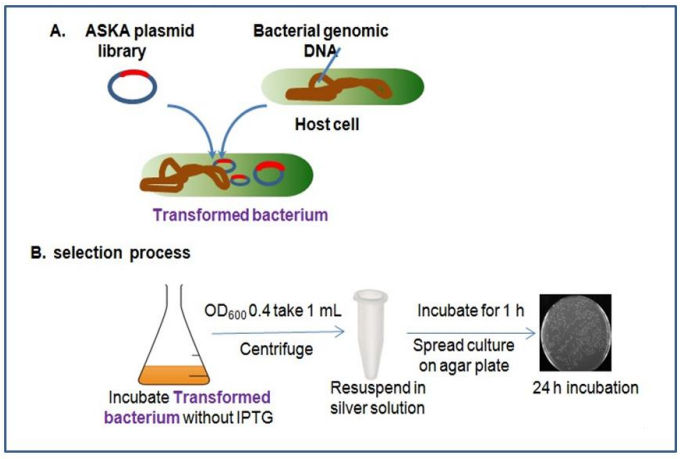
E. coli XL1-Blue cell was cultured in 5 ml of LB containing appropriate amounts tetracycline and glucose over night at 37°C in a shaking incubator. The next day, 100 µl of preculture XL1-Blue was inoculated into 10 ml of LB medium with tetracycline and then the culture was incubated at 37°C in a shaking incubator until the OD_{600} was around 0.4. 1 ml of sample was taken to centrifuge and the supernatant was discarded. Further, the pellet was resuspended in 200 µl of AgNPs or silver nitrate (Figure 2 shows the concentration of AgNPs and silver nitrate used for the MIC) and incubated for 1 h at 37°C with shaking, then 10 µl of culture was taken and diluted to 100 times. Finally 100 µl of dilution was spread to agar plate and the dishes were incubated upside down for 24 h at 37°C. MIC was assessed by checking CFU. When no growth of bacteria was observed in the medium containing

the lowest concentration of test materials, the MIC of the test material was defined at this point of dilution.

2.1.3 Selection of anti-silver genes in the absence and presence of IPTG

First, the pooled plasmids of the ASK library were transformed into *E. coli* XL1-Blue bacterium by electroporation (1.8 Kv, 0.1 cm cuvettes), then recovered with 1 ml of SOC medium containing tetracycline for 1 hour at 37°C in a shaking incubator (Scheme 2A). When getting the transformed bacterium, we collected all these colonies with LB medium and used the pool to inoculate 10 ml of LB medium with tetracycline and chloramphenicol in the absence or presence of 1 mM IPTG. The culture was incubated at 37°C in a shaking incubator until the OD_{600} was around 0.4, then 1 ml of sample was taken to centrifuge and the supernatant was discarded. Further, the pellet was resuspended in 200 µl of 100 and 200 µg/ml of AgNPs (chosen from the MIC testing) or silver nitrate solution and incubated for 1 h at 37°C with shaking like Sheme 1. Finally, 10 µl of culture was taken and diluted to 100 times. 100 µl of dilution was spread to agar plate and incubated for 24 h at 37°C (Scheme 2B). After getting surviving colonies, the second round of selection can be conducted by collecting the survival colonies from the first round of selection. Some single colonies were picked from the surviving colonies of pre-selection or non-selected and their DNA plasmids purified (by miniprep using a Machery Nagel kit) for DNA sequencing (Starseq AG, Germany or Microsynth, Switzerland).

Scheme 2. Schematic representation of process of anti-silver plasmid genes selection



2.1.4 Validation of anti-silver genes selected in the absence and presence of IPTG

The genes selected in the absence or presence of IPTG were individually retransformed by standard chemical method into *E. coli* XL1-Blue. Then a single colony of each transformed bacteria was picked and cultured in 5 ml of LB containing appropriate amounts of chloramphenicol and tetracycline over night at 37°C in a shaking incubator. The next day precultures were inoculated into LB medium with tetracycline and chloramphenicol in the absence or presence of 1 mM IPTG and incubated at 37°C in a shaking incubator until the OD_{600} was around 0.4 (incubated about 3 h with IPTG). A defined volume of culture was mixed with different types of silver and incubated for defined times at 25°C. Meanwhile 10 µl of sample was taken at different incubation times and

diluted 100 times with sterile MQ H_2O from which aliquots were spread on plates. The test materials AgNPs or metallic silver(0) preparations as well as silver ion supplied as silver nitrate were diluted when necessary with sterile MQ H_2O.

For the Live/Dead assay, after the strains induced by IPTG (OD_{600} 0.4) were incubated with silver ion or AgNPs for 3.5 h at 25°C, 1 ml of culture was taken to centrifuge and resuspended in 8.5% sodium chloride containing 0.3% Live/Dead mixed dyes (1:1): 300 µl for bacteria incubated with silver ion and 15 µl for AgNPs. The bacteria were then stained at 25°C for 10 min. After that, bacteria were centrifuged and resuspended in 300 µl or 15 µl of 8.5% sodium chloride solution. The images were acquired using a fluorescence microscope (AX10, Zeiss, Unispital Basel), and merged using Image J program. Live/Dead dyes provide a novel two-color fluorescence assay of bacterial viability: SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI). According the supplier's instructions, when used alone, the SYTO 9 stain generally labels all bacteria in a population — those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide.

2.1.5 Testing *cysD* overexpression in presence of Cd^{2+}, Al^{3+}, Pb^{2+} and Cu^{+}.

The selected gene *cysD* which gives the most resistance to silver also was tested with other different metal ions. This gene and the empty pCA24N vector were individually retransformed by chemical method into *E. coli* XL1-Blue. The experimental process is the same as for section 2.1.4 The ASKA strains culture with the resistant gene and empty control were incubated in the presence of IPTG at 37°C until the OD_{600} was around 0.4, then different concentrations and valences of metal ions ($CdCl_2$, $AlCl_3$, $Pb(NO_3)_2$, CuBr) were added and the cultures were incubated for 7 h at 25°C. 10 µl of sample was taken at different incubation times and diluted 100 times with sterile MQ H_2O from which aliquots were spread on plates.

2.1.6 Protein analysis and measurement of thiol groups in bacterial extracts.

ASKA strains were grown in LB medium containing tetracycline and chloramphenicol at 37°C under induction of IPTG or not. When OD_{600} reached 0.4, 1 ml of sample was taken as the control. Then silver solution was added to final concentration of 6 µg/ml for silver ion supplied as silver nitrate and the culture was incubated for 7 h at 25°C. One ml of sample was taken at 3.5 and 7 h for the analysis of proteins expressed with and without IPTG. To extract the protein, the pellet from the 1 ml of culture was lysed using lysis buffer containing 1 mM PMSF as well as DNAse l and was shaking at room temperature for 1 h. The lysate was centrifuged and the soluble part was analyzed by SDS-PAGE in order to measure the expression of proteins in the IPTG induction or not. To make polyacrylamide gels, the stock solution used was: 30% acrylamide, 1.5 M Tris-HCl buffer (pH 8.8), 20% sodium dodecylsulfate, 0.5 M Tris-HCl buffer (pH 6.8), 15% ammonium persulfate (APS) solution, and tetramethylethylenediamine (TEMED). In running gel polyacrylamide is 12%, and 5% in stacking gel. SDS-PAGE analyses were carried out according to Laemmli, and protein estimations followed standard protocols.[11]

Thiol groups were quantified by Ellman's assay: 2.5 ml of a reaction buffer containing 100 mM sodium phosphate (pH 8) and 1 mM EDTA was prepared, then 50 µl of a 5,5'-dithiobis-(2-nitrobenzoic acid) solution and 250 µl of protein lysis solution from the culture induced by IPTG (obtained according to the above method) was added into the above reaction buffer containing EDTA. The absorbance at 412 nm was measured after the mixture was incubated at room temperature for 30 min. A molar extinction coefficient of 14150 $M^{-1}cm^{-1}$ can be used to calculate the thiol concentration.

2.1.7 Comparison of silver resistance of selected genes under anaerobic and aerobic conditions or presence of H_2O_2

To explore the role of oxidative stress on antimicrobial activity of silver, we tested the anti-silver ability of the selected genes to silver under anaerobic and aerobic conditions, as well as in presence of H_2O_2. Cultures with 0.4 OD_{600} were induced with IPTG for 3 h, and one half degassed with nitrogen for about 30 min; then the metallic silver(0) and silver ion were added to the cultures by injection. Meanwhile H_2O_2 was added to another set of culture with oxygen. The cultures with and without oxygen were incubated at 25°C for defined times. Samples (10 µl) were taken at different incubation times and diluted 100 times with sterile MQ H_2O, from which aliquots were spread on plates.

2.1.8 Measurement of OH• produced by Ag^+ induction

Hydroxyl radicals (•OH) have been suggested to be a crucial component of apoptosis. The HPF (3′-(p-hydroxyphenyl) fluorescein) assay was used to measure intracellular accumulation of hydroxyl radicals. Non-fluorescent HPF can be oxidized by hydroxyl radicals to a fluorescent derivative. The cultures were incubated for 3 h after adding IPTG until OD_{600} is ~0.4. Then 1 ml of culture was added to the corresponding concentration of silver ion and HPF (final concentration 5 µM; 0.1% dimethylformamide as a cosolvent). Samples were incubated at 25°C for 1 h and then centrifuged to remove excess. The bacterial pellet was resuspended with 1×PBS buffer, PH 7.4. The samples were analysed with a fluorescence microscope (AX10, Zeiss) or Tecan Flurimeter. The fluorescence intensity was measured at 515 nm with excitation at 490 nm.

2.1.9 The synergistic effect of antibiotics and silver on anti-silver genes

To test effects of antibiotics on selected strains, a similar experimental process was used as with silver resistance tested on agar plates (see section 2.1.4). To determine any synergistic effects, different concentration of antibiotics (ampicillin and kanamycin) and silver as well as the combination of both were added to the 0.4 OD_{600} of culture induced by IPTG and incubated for 7 h at 25°C. Samples (10 µl) were taken at 3.5 and 7 h and diluted 10 times with sterile MQ H_2O, from which 4 µl of dilution was dropped on agar plate.

2.2 Results

2.2.1 The effect of size and concentration of silver and reaction time on antibacterial activity—*E. coli*

In order to research the effect of concentration of silver and size of AgNPs on bacteria, we used *E. coli* XL1-Blue bacteria (without any transformed plasmid) to test the MIC. The experimental process is shown in Scheme 1. In general, bacteria incubated in the presence of added silver ion had less survival than those subjected to 10 and 50 nm AgNPs, at the same concentration of silver content (Figure 2). Even at the concentration of silver ion as low as 5 μg/ml, only about 40% survived after 20 minutes incubation. In contrast, bacteria in the presence of AgNPs have high survival, even at 50 μg/ml, that is 10 times higher than the MIC of silver ion, underscoring that the antibiotic ability of AgNPs is relatively weak compared to silver ion. However, antimicrobial activity of AgNPs was dose-dependent from 20 μg/ml, where survival of bacteria is almost the same as control, to 400 μg/ml, at around the MIC Activity was also time-dependent: in all cases, when bacteria were treated with silver for 60 min, less bacteria survived at the same concentration compared to 20 min treatment. Finally, the antimicrobial activity of nanoparticles was also size-dependent: bacteria incubated with 10 nm AgNPs had less survival than 50 nm AgNPs. For example, 10 nm AgNPs had 20 times less survival than 50 nm AgNPs at the concentration of 100 μg/ml. The results demonstrated silver ion has much more antibiotic activity than AgNPs. In our experimental conditions, the MIC of silver ion and 10 nm AgNPs is 20 μg/ml and 400 μg/ml respectively. For the selection of resistant genes, we chose conditions close to the MIC based on these preliminary findings, namely incubation for 1 h, with 100 and 200 μg/ml AgNPs and 5 μg/ml silver ion.

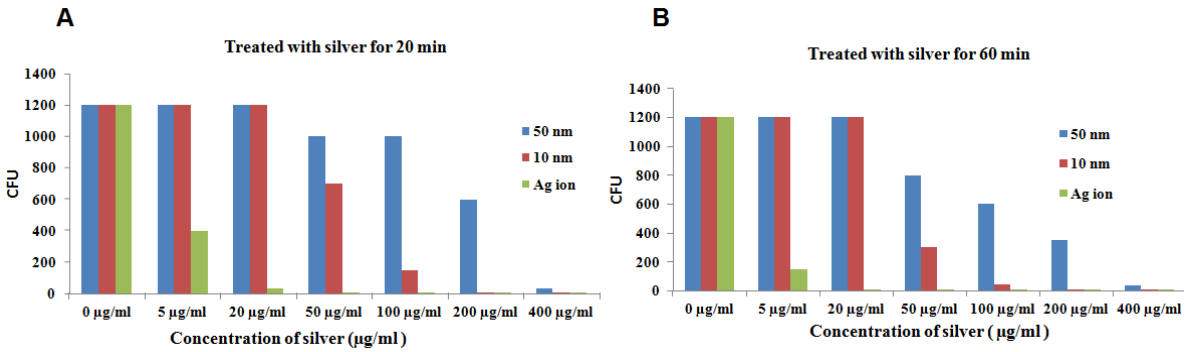


Figure 2. The effect of concentration of AgNPs and silver nitrate on the antimicrobial activity. Bar graphs compared to the antimicribiol activity of 50 nm, 10 nm and silver ion under a series of concentrations from 0 to 400 μg/ml. the bacteria was incubated 20 mins (A) and 60 mins (B) after adding silver ion and AgNPs.

2.2.2 Selection of anti-silver genes in the absence and presence of IPTG

Selection of anti-silver genes in the absence of IPTG

We initially selected anti-silver genes in the absence of IPTG, using a stringent selection in low-level constitutive expression of cloned proteins. In a first round of selection many clones survived using 50 nm AgNPs (40 colonies). In contrast, less and no ASKA strains survived in presence of 10 nm

AgNPs (14 colonies) and silver ion (no colonies) under 100 µg/ml of condition, which reinforced the notion that large nanoparticle size of AgNPs leads to lower toxicity than small size. Similar results were found in subsequent experimental replicates. A second round of selection was conducted using all strains surviving from the first round of selection, leading to more colonies surviving for the "pre-selection pool" than the "no selection pool" for 50 nm AgNPs (Table 1). The result that pre-selection can give increased numbers of survivors supports the view that silver-resistance is genetically-encoded by the plasmids. Since the selected ASKA strains gave more resistance to AgNPs, we picked and sequenced the inserts of some of the selectants (Table 1). In the pre-selection with10 nm AgNP both sequenced genes were identified as *iscA*, encoding a sulfur-iron protein (see Chapter 4). The other 3 sequenced ASKA plasmids selected at a replicate experiment were *yphH*, *gpH* and *ycdB.* Surprisingly, all seven sequenced colonies from a second round of selection were found to harbour *ydgT*, which indicates that *ydgT* may confer higher resistance to silver than other ASKA plasmid genes in the absence of IPTG.

Table 1. The selection conditions and sequencing results of anti-silver genes using *E. coli* XL1-Blue bacteria containing ASKA plasmids in the absence of IPTG.

Solution	**Ag concentration**	**ASKA-1 (CFU) (preselected with 10 nm AgNP)**	**ASKA-2 (CFU) (no preselection)**
10 nm AgNPs	100 µg/ml	No survivors	No survivors
	200 µg/ml	No survivors	No survivors
50 nm AgNPs	100 µg/ml	25 (pick 6)	3(pick 3)
	200 µg/ml	1 (pick 1)	No survivors
Silver ion	100 µg/ml	No survivors	No survivors
	200 µg/ml	No survivors	No survivors

Sequences	**Gene**
ASKA-1, 2st round of selection	*ydgT* [a] (7 copies)
ASKA-2, no preselection, 50 nm, 100 µg/ml	*ycdB*[b], *yphH* [b], *gpH* [b]
ASKA-1, 1st round of preselection (pre-selection)	*iscA*[c] (2 copies)

a: Two rounds of selection
b: Replicate of 1st round of selection
c: In the pre-selection with 10 nm AgNP (1st round of preselection) 2 clones sequence were identified as *iscA*, which is researched further in chapter 4.

Selection of anti-silver genes in the presence of IPTG

Table 2. The selection conditions and sequencing results of anti-silver genes using *E. coli* XL1-Blue bacteria containing ASKA plasmids in the presence of IPTG.

Ag concentration	**CFU**
100 µg/ml AgNPs (10 nm)	~250
200 µg/ml AgNPs (10 nm)	~200 (picked 20 colonies)
5 µg/ml Ag ion	20 (picked 20 colonies)
10 µg/ml Ag ion	no
20 µg/ml Ag ion	no

Sequences Gene
yeaO (4 from AgNPs, 2 from $AgNO_3$)
cysD (5 from AgNPs, 14 from $AgNO_3$)
yabQ (2 from $AgNO_3$)
Other 13 plasmids: *thiQ, insA, yihD, cheY, ygeK, gcyT, ygiG, aroB, flhD, yceQ, yjdI, yraN, hofM*

Due to ASKA plasmid genes can be overexpressed under IPTG induction and overexpression of proteins may be able to impart more resistance to silver, we further selected the anti-silver genes from the ASKA library using IPTG with 1mM as inducer, using stringent selection conditions: Ag ion and 10 nm of AgNPs, which had higher antibacterial activity than 50 nm AgNPs. More strains survived under induction of IPTG compared to the condition without induction of IPTG at the same concentrations of AgNPs (Table 2), suggesting that protein overexpression leads to more resistance. For silver ion, strains only survived at 5 µg/ml, which confirmed that the toxicity of silver ion is much higher than for AgNPs. New candidates for silver resistance genes with IPTG induction were identified, such as genes *cysD, yeaO and yabQ*. Interestingly, 19 out of 40 colonies sequenced harboured *cysD*, 5 of which were from selection with AgNPs and 14 were from $AgNO_3$ selection. This result suggests strongly that *cysD* confers high-level resistance against silver and, further, that it exerts cross-resistance to both AgNPs and silver ion. Plasmids with *yeaO* were also selected from both AgNPs and $AgNO_3$, which also indicates cross-resistance mechanisms of *yeaO*. In addition, two copies of *yabQ* were selected, both from $AgNO_3$.

2.2.3 Validation of selected anti-silver genes

Validation of cross-resistance to silver ion

In order to validate the anti-silver ability of the selected genes, a negative control strain harbouring an empty pCA24N vector and a positive control *cueO*[10] were chosen to compare the anti-silver ability with the selected genes in the absence and presence of IPTG. The positive control, *cueO*, is a multicopper oxidase with a methionine-rich segment, which can bind to silver ion.[12] The empty pCA24N was used for the negative in order to ensure the same growth condition for the strains. The anti-silver ability was determined by either plate-based or broth-based methods.

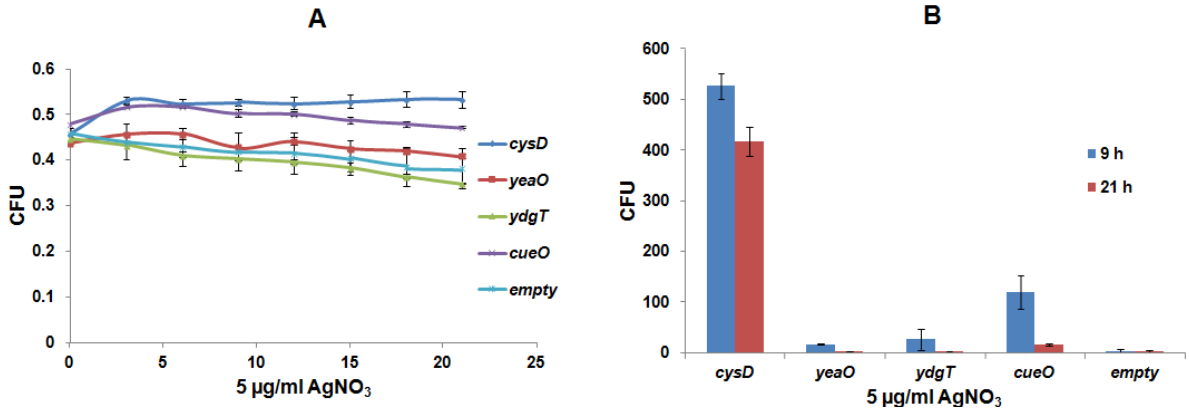


Figure 3. (A) Growth curve of strain with selected plasmids and control strains (*cueO* and empty as positive and negative control, respectively) during exposure to 5 µg/ml of silver ion for 21 h at 25°C in LB medium. (B) count of colony forming units (cfu) of strains incubated with silver ion for 9 and 21 h in LB in the presence of IPTG. The results represent the averages of three replicates with standard deviations.

To measure the anti-silver ability of these selected genes after adding silver, first the three genes identified in different clones, *cysD, yeaO* and *ydgT* were chosen for further validation. A broth-based method, in which 5 µg/ml of $AgNO_3$ were added to ASKA strains induced by IPTG (OD_{600} 0.4),

followed by monitoring OD_{600} at 25°C for 21 h, suggested that *cysD* and *cueO* had the most survivors. The plate-based assay confirmed these results, showing that when strains were incubated with silver ion for 9 h, the survival of strain *cysD* is much higher than other strains even the positive strain *cueO*. After 21 h of incubation, survival of *cysD* is still high but other strains almost have no surviving colonies.

Validation of cross-resistance to silver ion and AgNPs

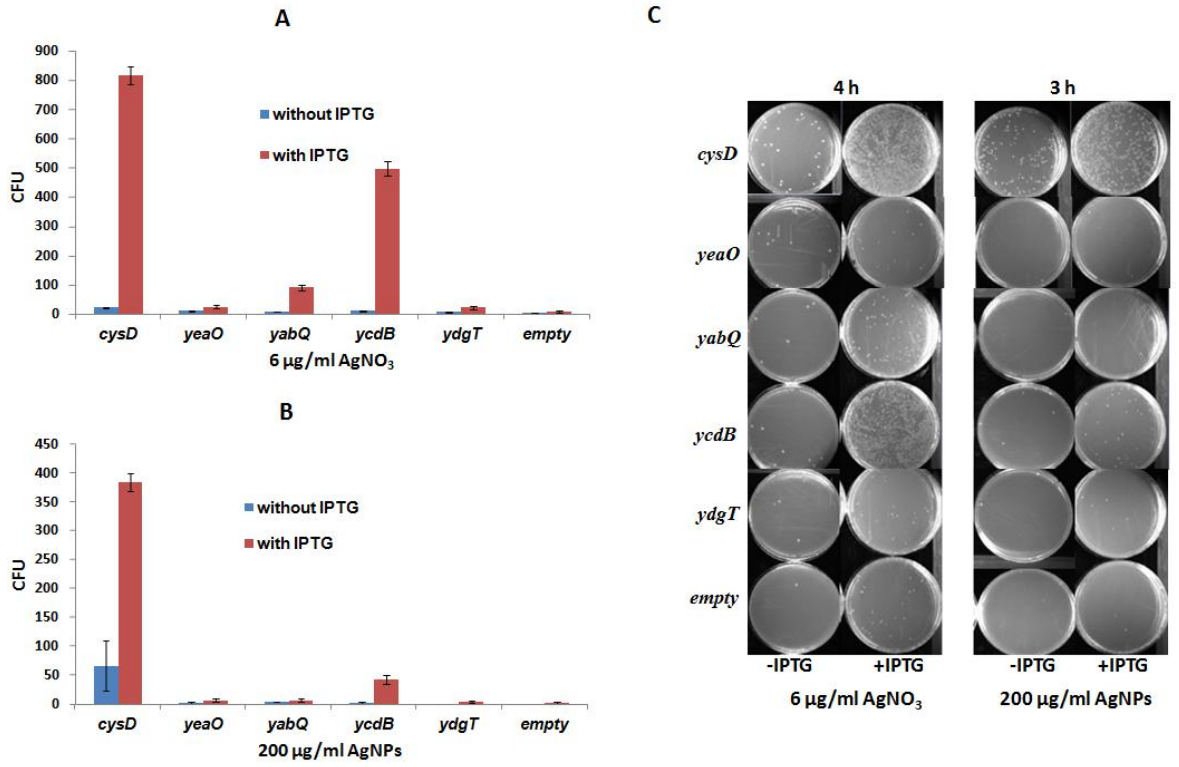


Figure 4. Comparative analysis of the anti-silver ability of strains selected (A) Bar graphs of bacterial count for 6 µg/ml $AgNO_3$ and (B) 200 µg/ml AgNPs in the presence or absence of IPTG after 4 and 3 h of incubation. (C) corresponding petri dishes of strains incubated with 6 µg/ml $AgNO_3$ and 200 µg/ml AgNPs. The results represent the averages of three biological replicates with standard deviations.

Further, we compared anti-silver ability of the selected genes by incubating the selected strains in the presence of Ag ion or 10 nm AgNPs as antimicrobial agents in the presence or absence of IPTG (Figure 4). Since *yphH* and *gpH* selected without IPTG did not show more resistance to silver than control (data not shown), these two genes were not studied further. The other 13 single genes selected with IPTG were not verified further because they were found only in single clones, suggesting lower resistance. In these validations, more colony forming units were also present with IPTG induction than without, which indicates that higher protein expression is related to resistance. There were many more survivors with strains *cysD* and *ycdB* than with all other strains tested, for both types of silver after 4 and 3 h of incubation with silver in the presence of IPTG (Figure 4A and B). However, *yeaO*, *yabQ* and *ydgT* also exerted moderate resistance to silver under induction of IPTG, compared to the empty control. The order of resistance ability of all strains to silver ion and

AgNPs was coincident, that is, strains *cysD* and *ycdB* have higher resistance to silver ion and AgNPs compared to other strains. The expression of *cysD* gave by far the higher resistance. The much higher toxicity of Ag ion and the anti-silver ability of the selected ASKA genes regardless of the form of silver are consistent with the notion that silver nanoparticles exert their antimicrobial activity chiefly by releasing Ag ion.

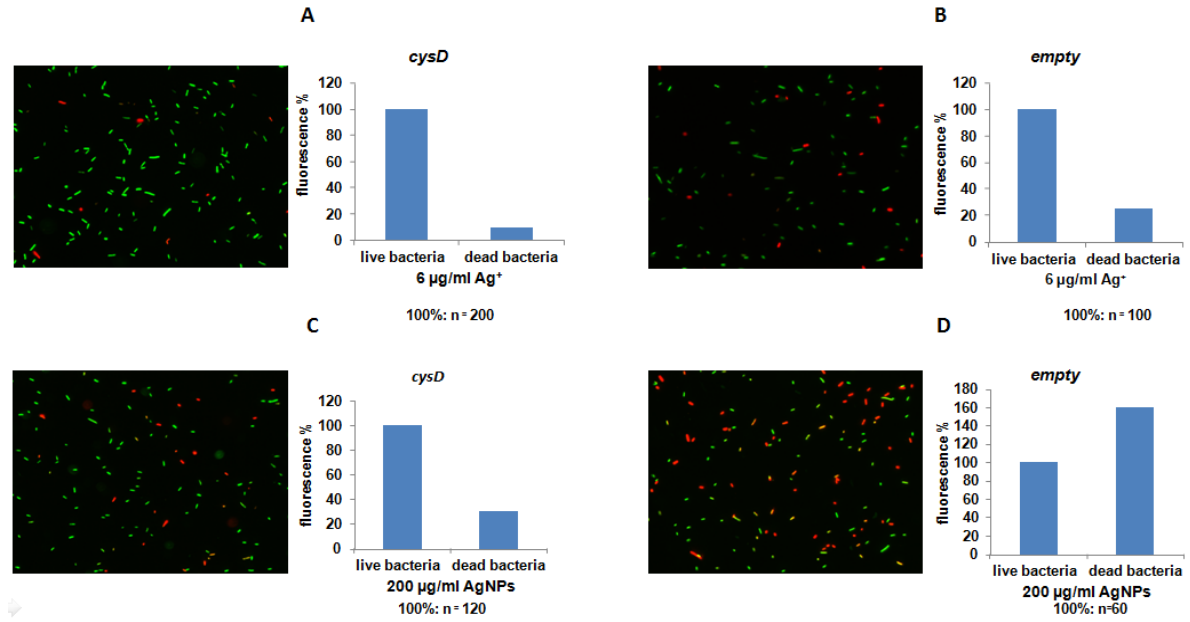


Figure 5. Fluorescence microscopy of strain *cysD* (A) and (B) empty treated with 6 μg/ml Ag ion; strain *cysD* (C) and (D) empty treated with 200 μg/ml AgNPs for 3.5 h using Live/Dead dyes. The bar graphs display the number ratio of live/dead bacteria from two pictures. n represents the number of live bacteria ,which serves as 100% standard.

Treatment with some antibiotics or with silver can lead to increased cell length[13] and increase the permeability of membrane.[3(c)] We asked whether silver can prompt bacteria to elongate and increase the permeability of membrane in the different selected strains. We also want to detect the viability and morphology of strains harbouring *cysD* or the empty plasmid control. Comparison of the cell morphology of silver ion or AgNP-treated strains *cysD* and empty revealed no obvious differences by microscopy indicating that silver did not induce large changes in the shapes of strains (Figure 5), such as cell-elongation, which has been observed upon treatment with silver or certain antibiotics.[13] However, visualization and quantification of cells stained with SYTO 9 stain (green color, indicating viability) and PI stain (red color, indicating membrane damage) confirmed increased viability and less membrane damage for the *cysD* strain compared to the empty control, both in presence of silver ion and AgNPs. These results are consistent with other forms of validation of increased *cysD* resistance to silver, such as quantification of colony-forming units.

Cross-resistance to other types of silver

We also explored resistance of *cysD* and *ycdB* to commercial metallic Ag(0) stabilized in gelatine and Ag ion colloid (kindly provided by Nanotrade), to confirm the universality of resistance of these two genes to different kinds of silver of commercial relevance (Figure 6). Consistent with previous results with $AgNO_3$ and with citrate-stabilised AgNPs, both *cysD* and *ycdB* had increased resistance

compared to empty control, using 10 µg/ml of Ag ion colloid and 55 µg/ml metallic Ag (bar graph from Figure 6A and B). As previously found, *cysD* exerted the greatest resistance also for these alternative antimicrobial silver preparations.

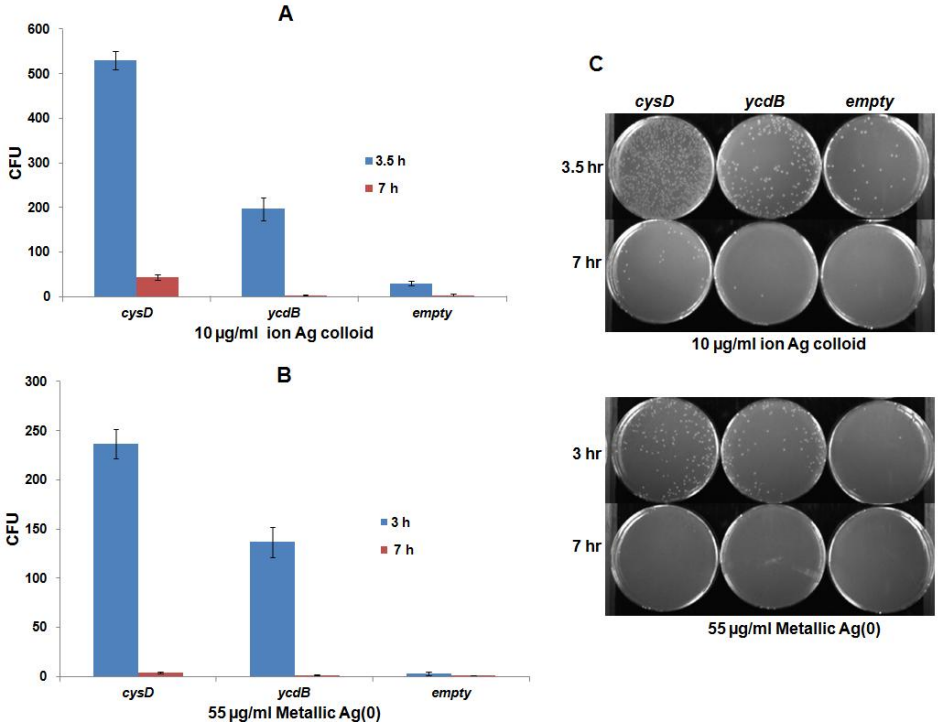


Figure 6. Comparative analysis of strains *cysD* and *ycdB* to metallic Ag(0) stabilized in gelatine and Ag ion colloid. (A) and (B) Bar graphs of bacterial count and (C) petri dishes of strains incubated with 10 µg/ml Ag ion colloid and 55 µg/ml metallic Ag(0) for 7 h with IPTG induction. The results represent the averages of three biological replicates with standard deviations.

2.2.4 Cross-resistance of *cysD* to Cd^{2+}, Al^{3+}, Pb^{2+} and Cu^{+}

Ag(I) and Cu(I) ions are both soft metals and have the same outer electronic configuration, with a closed-shell structure (d10), and similar binding properties to ligands such as proteins. Copper detoxification systems in general, bind and transport both Ag(I) and Cu(I). For example, Ag(I) binds CueO at the same sites like Cu(I) substrate ions in CueO;[14] CueR[15] can bind and transport Cu(I) and Ag(I) cargos at the same sites. In isolated membrane vesicles, CopB pumped Ag^{+} and Cu^{+} at similar rates and with similar affinities.[16] Other observations also suggest that copper ATPases can pump silver, such as CopA of *Archaeoglobus fulgidus*, which was shown to be more strongly activated by silver(I) than by copper(I).[17] The possibility for combined Cu(I)/Ag(I) stress is common.[18] On basis of the fact that Cu(I)-binding sites in proteins can often bind Ag(I), we hypothesized the possibility that anti-silver genes may be also exert cross-resistance to Cu(I). In addition we explored the extent of specificity compared with other metal ions. Cd^{2+}, Al^{3+}, Pb^{2+}, and Cu^{+} were chosen to test antimicrobial ability of strain *cysD* (Figure 7). Under the conditions tested, both Al^{3+} and Pb^{2+} were only moderately toxic and no significant differences in survival were found between the *cysD* and *empty* strains (data no shown). However, clear differences were found both for CuBr and $CdCl_2$ indicating that *cysD* also provided some cross-resistance to Cu^{+} and Cd^{2+}.

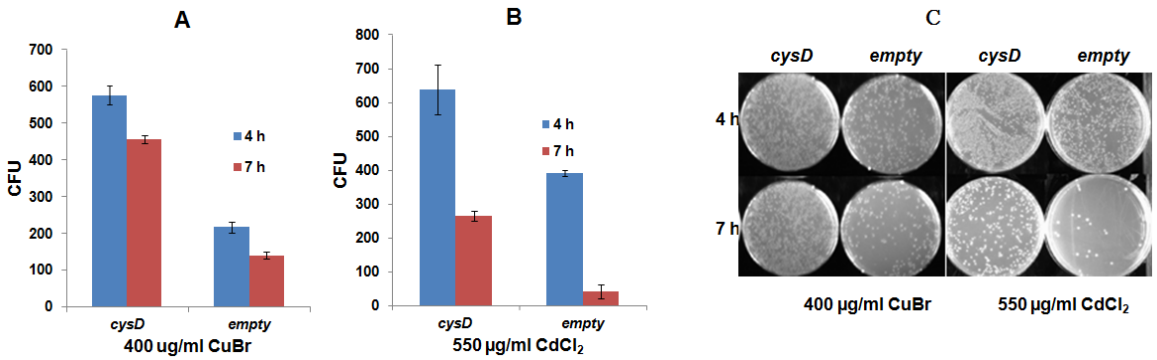


Figure 7. The increased ability of strain *cysD* to survive in presence of different metal ions. (A) and (B) Bar graphs of bacterial count surviving in the presence of Cu^{+} and Cd^{2+}. (C) corresponding representative petri dishes of strains incubated with 400 µg/ml Cu^{+} and 550 µg/ml Cd^{2+}. The results represent the averages of three biological replicates with standard deviations for Cu^{+} and Cd^{2+}.

2.2.5 SDS-PAGE analysis of protein extracts.

Analysis of cell extracts of *cysD*, *ycdB*, *cueO* and "*empty*" control by SDS-PAGE/ coomasie staining (Figure 8) revealed the following :

- Level of expression of CysD (35.203 KDa) was not visible for all different experimental conditions tested, compared to strain empty, indicating the expression of CysD protein is very low even under induction of IPTG.
- For YcdB, very little protein is detected in the soluble cell extracts at approximate 48.3 KDa under induction of IPTG and without addition of silver ion, which corresponded to the full-length intact polypeptide YcdB. YcdB and CueO genes both contain Tat signal sequence, so both proteins can be translocated to the periplasm by the Tat export pathway, after which Tat signal sequences are cleaved to form a mature protein (mature protein). Only YcdB with Tat signal sequence (pre-YcdB) could be detected, but no mature YcdB for all conditions tested, indicating expression of YcdB in the periplasm was very low.
- For CueO there were two obvious bands corresponding to the size of 58.4 KDa and 55.4 KDa for all conditions tested, upon induction with IPTG. These two bands correspond to pre CueO and mature CueO (mat-CueO). The presence of these two obvious bands also indicates CueO was overexpressed well and that the addition of silver ion had no effect on the expression of CueO with IPTG induction. There was no band corresponding to CueO protein in the absence of IPTG, which indicates that IPTG induction was effective for increased protein expression.

It is particularly noteworthy that the expression of CueO and YcdB protein was apparently much higher than that for CysD, which was not detectable on SDS-PAGE analysis, although the silver-resistance of *cysD* was much higher, thus suggesting that *cysD* can detoxify silver ion very effectively, perhaps even catalytically.

2.2.6 Measurement of thiol groups in cell extracts.

CysD is an enzyme and a component of the cysteine-metabolic pathway,[19] which indicated a possible silver-resistance mechanism. The amount of thiol groups in selected ASKA strains were quantified in cell extracts of selected strains to explore whether increased levels of this potential sulfur-donor ligand correlated with increased survival in presence of silver. Extracts of the ASKA strain *cueO* contained marginally more thiol groups than other ASKA strains, although this was not statistically very significant, while CysD had the least thiol amount. However, these minor differences were not significantly different, indicating that thiol amounts were probably not the main reason for the generation of anti-silver ability of *cysD*.

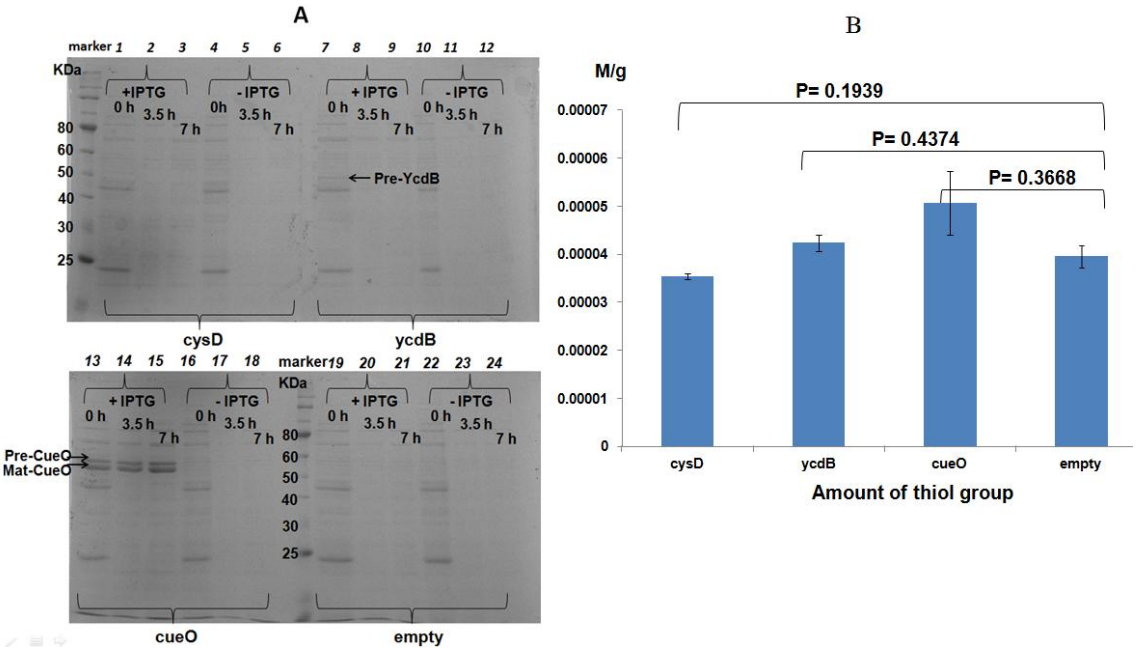


Figure 8. (A) SDS–PAGE analysis with Coomassie blue staining of whole cell proteins from different ASKA strains at different incubation with 6 µg/ml silver ion with and without IPTG. Molecular mass markers are indicated in the middle and the left of gel. (B) The amount of thiol group of each strain overexpressed with IPTG. Error bars represent means SD (standard deviation) from three biological replicates. P <0.1939, P <0.4374, P <0.3668, P <0.4156, Student's test. Pre (pre protein), Mat (mature protein).

2.2.7 Comparing the anti-silver ability of selected strains under anaerobic and aerobic conditions

Nanosilver particles have been reported to generate the biological activity by silver ion release,[20-22] but there remains some controversy about the importance of these particle-based mechanisms.[9,23] In order to research the mechanism of AgNPs toxicity and to explore the effect of oxygen on antibacterial activity of AgNPs and silver ion, we compared the anti-silver ability of ASKA strains *cysD* and *ycdB* to AgNPs under anaerobic and aerobic conditions (Figure 9A). It has been suggested that AgNPs can release silver slowly in the presence of dissolved oxygen and protons in the surrounding fluid phase, by oxidation of Ag(0) to Ag(I) from the surface of AgNPs.[20] We postulated that if the biological activity of AgNPs is achieved by particle-based mechanisms, strains expressing

CysD could have similar survival rates under anaerobic and aerobic conditions. The number of surviving clones for *cysD* when incubated with AgNP under anaerobic conditions was about 4 times higher than when tested under aerobic conditions. Bacterial killing by Ag^+ was found to be oxygen-dependent with incubation time, with different colony forming units (cfu) found in aerobic and anaerobic conditions under the conditions tested. Increased resistance of *cysD* against silver ion under anaerobic conditions is likely to arise from reduced oxidative stress involved in the antibiotic mechanism of silver.

Reaction 1: $2Ag(s) + 1/2O_2(aq) + 2H^+ \longrightarrow 2Ag(I)(adsorbed) + H_2O(l)$

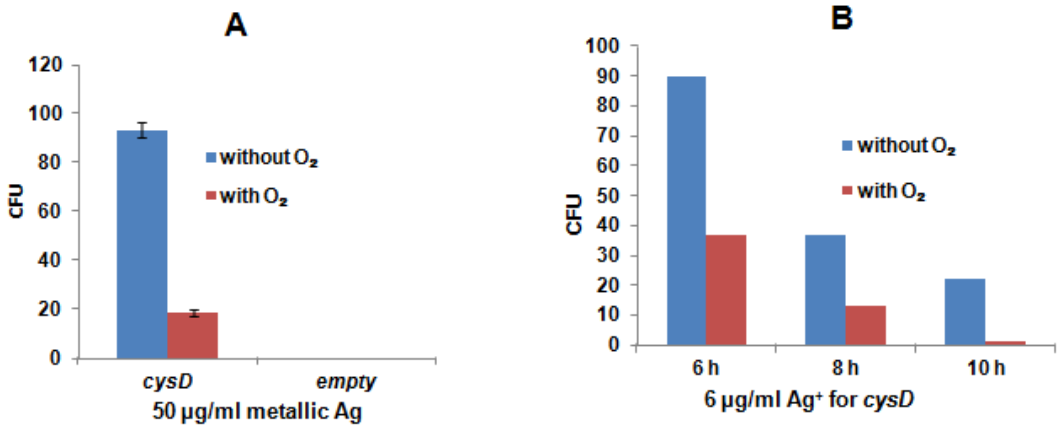


Figure 9. The resistance ability of strain *cysD* to metallic silver and silver ions under anaerobic and aerobic conditions. (A) Bar graphs of bacterial count surviving in the presence of 50 µg/ml metallic silver after 6 h of incubation (B) and 6 µg/ml Ag^+. Error bars represent means SD (standard deviation) from three biological replicates.

The increased resistance of *cysD* against AgNPs (and other strains tested, see chapter 3) in anaerobic conditions compared to anaerobic conditions is compatible with the notion that oxidation and release of ionic silver (Ag^+) is a major mechanism of antimicrobial killing (Figure 9A). These results suggest that under depleted oxygen AgNPs cannot be further oxidized and silver ion is not produced, leading to low toxicity of the particles. However, it is noteworthy that AgNPs were toxic to bacteria even in anaerobic conditions. Multiple silver species may coexist in AgNPs solution, including particulate zero-valent silver (Ag(s)), surface oxidized silver (Ag(I)-(adsorbed), reaction 1a), and dissolved silver cations (Ag(I)-(aq), reaction 1b);[22] we did not exclude the possibility that the toxicity exerted by the AgNPs in anaerobic conditions originates in forms of silver ion that potentially existed in the AgNPs stock solution.

The increased susceptibility of *cysD* to silver ion under aerobic compared to anaerobic conditions suggested that oxidative stress is involved in the antibiotic mechanism of silver ion. In order to confirm that effect of exogenous oxidative stress on the resistance of strain expressing CysD, we compared their resistance to H_2O_2, which can product reactive oxygen species. The results demonstrated that antimicrobial activity of H_2O_2 is concentration-dependent (Figure 10A). However, *cysD* had similar resistance to H_2O_2 as the empty control, suggesting that *cysD* does not exert a protective activity against exogenous oxidative stress caused by H_2O_2 (Figure 10B). Therefore, it

appears that *cysD* does not have a general protective mechanism against reactive oxygen radicals (ROS).

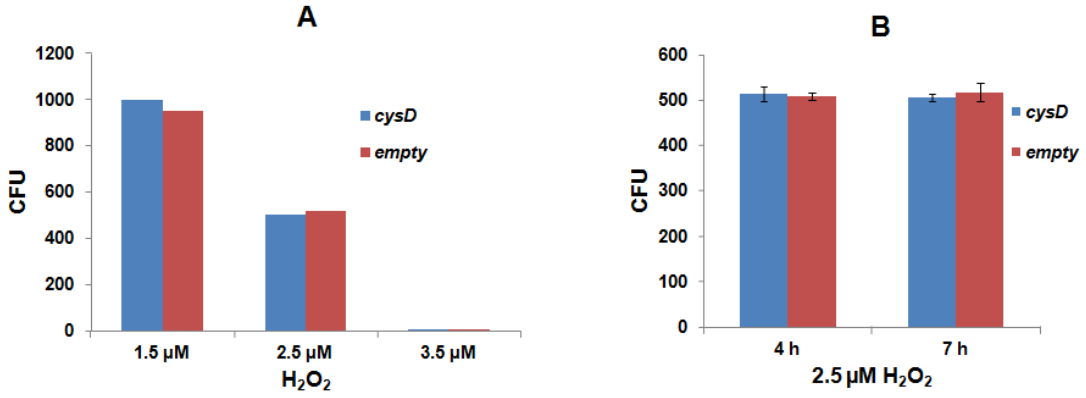


Figure 10. The resistance ability of strain *cysD* to H_2O_2. (A) Bar graphs of bacterial count surviving in the presence of different concentration of H_2O_2 after 4 h of incubation (B) Bar graphs of bacterial count surviving in the presence of 2.5 µM of H_2O_2 after 4 and 7 h of incubation. Error bars represent means SD (standard deviation) from three biological replicates.

2.2.8 The measurement of OH• produced by Ag^+ induction

It has been shown that silver may act as a catalyst for the production of hydroxyl radicals in the Fenton reaction.[24] Accumulation of ROS may lead to death of bacteria.[24] In order to research the resistance mechanism of bacteria to silver we determined the production and accumulation of intracellular hydroxyl radicals by using the fluorescent dye HPF (3′-(p-hydroxyphenyl) fluorescein) under the condition of addition of silver (Figure 11). By fluorescence microscopy, the empty control strain presented more fluorescent bacteria with increasing concentrations of silver ion from 2 to 6 µg/ml, indicating increased •OH production (Figure 11A), which supports that silver ion can induce the production of •OH in bacteria. When concentration of silver ion increased to 10 µg/ml the fluorescence did not further increase with concentration of silver ion compared to 6 µg/ml silver ion, but actually decreased a little, which we ascribe to excessive killing of bacteria under these conditions, which lowers the number of bacteria producing •OH. HPF fluorescence of the empty control strain and CysD-expressing strain was quantified in cell suspensions by spectrometry, which revealed similar silver-dependent trends. These measurements revealed that *cysD* consistently had lower fluorescence than the control strain, even at basal level, *i.e.* without addition of silver nitrate. A similar behaviour was found for the *ycdB* strain, which like *cysD* also had lower fluorescence compared to the empty control strain, even in the absence of Ag^+. All these results suggest that silver ion can disrupt metabolic pathways, leading to increased Fenton chemistry and overproduction of hydroxyl radicals, and that *cysD* and *ycdB* may be protecting against Ag^+-mediated cell-killing through prevention of formation of •OH-radical. This scenario is consistent with the observation that resistance of *cysD* to Ag^+ was also oxygen-dependent (Figure 9), this is, silver ion can induce the production of hydroxyl radicals, particularly in the presence of oxygen.

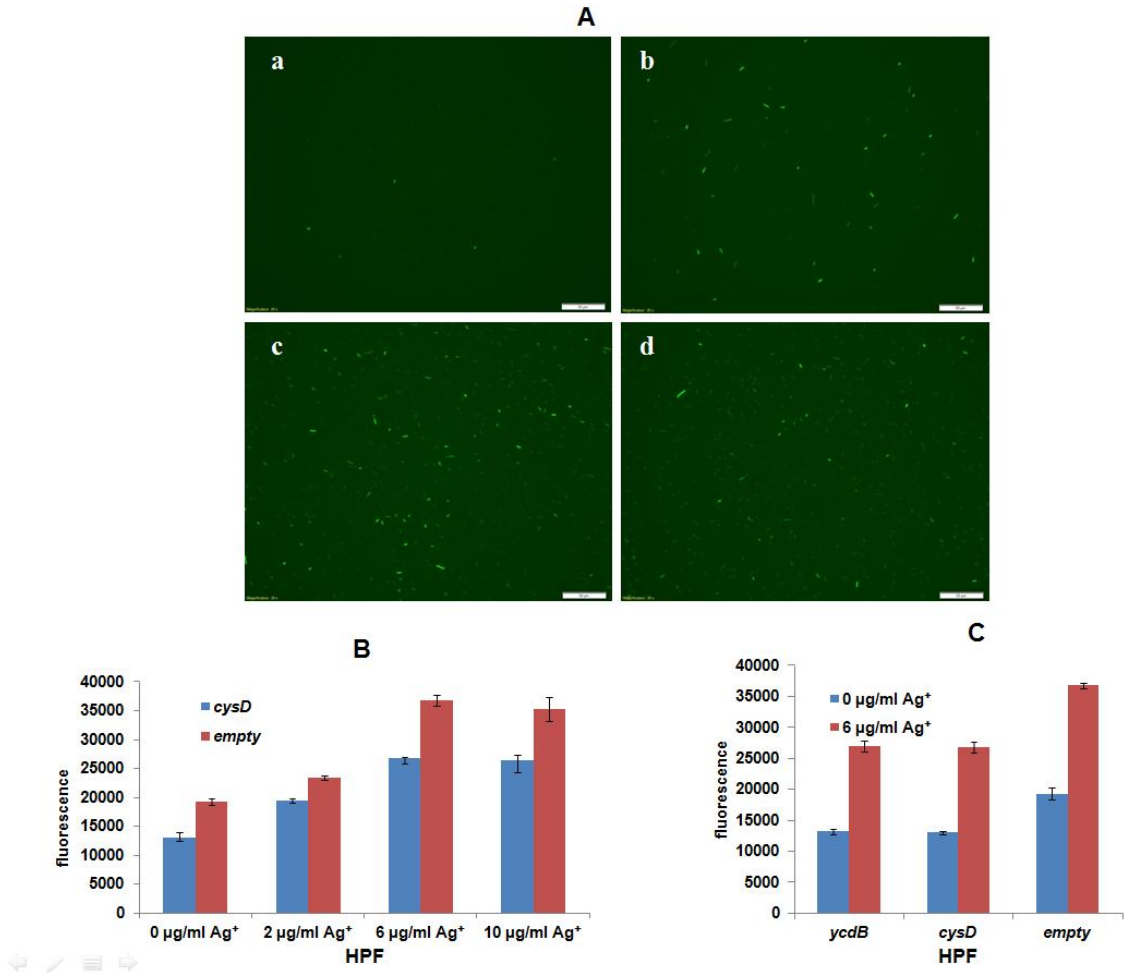


Figure 11. •OH production induced by Ag^+ in the Fenton reaction. (A) fluorescence microscopy of HPF-stained 1 h of strain empty cells with (a) 0 μg/ml Ag^+; (b) 2 μg/ml Ag^+; (c) 6 μg/ml Ag^+; (d) 10 μg/ml Ag^+; (B) fluorescence histogram of strains *cysD* and *empty* cell dyed by HPF for 1 h with different concentration of Ag^+. (C) fluorescence histogram of strains *ycdB*, *cysD* and empty cell dyed by HPF for 1 h with 0 μg/ml Ag^+ and 6 μg/ml Ag^+. Scale bar in Figure A is 50 μM.

2.2.9 The synergistic effect of antibiotics and silver in bacteria expressing silver-resistance genes

It has been suggested that AgNPs and silver ion can increase the antimicrobial activity of antibiotics.[3] Therefore, we asked the question whether the anti-silver genes we identified could also give rise to resistance to some antibiotics. To investigate the effect of silver-resistance genes on the potential synergistic antimicrobial effect of silver combined with antibiotics we chose to investigate combinations of silver and antibiotics that were sub-lethal when used alone (Figure 12). Neither 6 μg/ml kanamycin nor 2 μg/ml silver ion had much antimicrobial effect on the strains *cysD* and *ycdB* after incubation of 3.5 h and 7 h. In contrast, kanamycin had evident antimicrobial activity against the empty control strain after incubation of 7 h, indicating that the selected genes *cysD* and *ycdB*, selected for silver-resistance, also gave rise to resistance to the antibiotic kanamycin. Moreover, the combination of 6 μg/ml kanamycin plus 2 μg/ml silver ion had a marked antimicrobial activity,

including against strains *cysD* and *ycdB*, achieving increased antibacterial efficiency compared to the individual antibacterial effect of kanamycin and silver ion (Figure 12A). Similar synergism in antibiotic activity between silver and kanamycin was also observed when using AgNPs (Figure 12B): when 2 µg/ml kanamycin and 50 µg/ml AgNPs were used separately there was no obvious antibacterial activity observed, but when applied together, a marked antimicrobial activity was evident. Importantly, neither *cysD* nor *ycdB*, both of which protect against silver-mediated killing, appeared to exert any significant protective effect against the combined effect of silver ion and kanamycin under the conditions tested.

In presence of ampicillin, in contrast to the synergistic effect of the combination of silver and kanamycin, no synergy was found with silver antibiotic activity (Figure 12C): the combination of silver ion at 5 µg/ml with ampicillin at 24 µg/ml did not lead to increased bacterial killing. We did not exclude the possibility that ampicillin, which contains both sulfur and carboxyl groups, could itself bind to silver ion. However, it is noteworthy that *cysD*, which was selected for increased resistance to silver, also rendered bacteria more resistance to ampicillin, as well as to the combination of silver nitrate and ampicillin, compared to the control strain (Figure 12C).

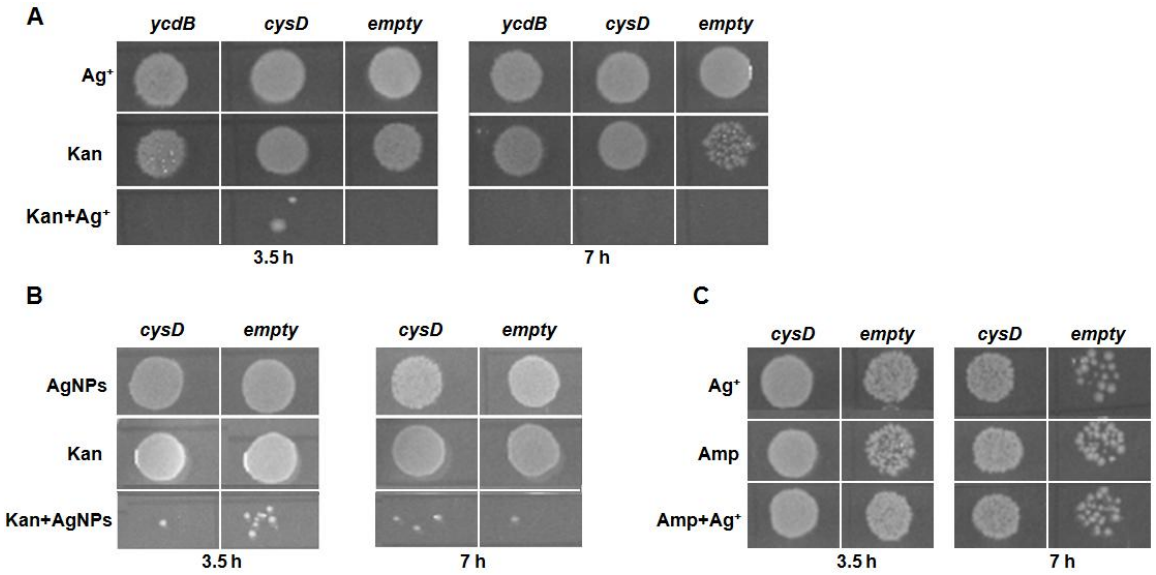


Figure 12. The resistance ability of strains *cysD* and *ycdB* to silver and antibiotics. (A) Strains treated with 2 µg/ml Ag^+, 6 µg/ml kanamycin or a combination of both. (B) Strains treated with 50 µg/ml AgNPs, 2 µg/ml kanamycin or a combination of both. (C) Strains treated with 5 µg/ml Ag^+, 24 µg/ml ampicillin or a combination of both. Amp-ampicillin, Kan-kanamycin.

To explore whether the synergistic effects of kanamycin and silver were due to the increased permeability of the bacterial membranes, we used propidium iodide (PI) to detect permeation of the cell membrane in the presence of antibiotics and silver (Figure 13A). The combination of kanamycin and silver led to an increase of fluorescence of both strains tested (*cysD* and *empty* control) compared to either antimicrobial agent alone, suggesting increased permeability of the strain membrane leading to increased bacterial killing. In contrast, the level of fluorescence of strains treated with silver in combination with ampicillin did not increase compared to either treatment

alone (Figure 13B), indicating ampicillin and silver had no synergistic effects on cell permeability. These results are consistent with the cell-viability assays (Figure 12).

To examine the effect of combination of silver ion and antibiotics on the production of •OH, the combined effect of silver ion and antibiotics was examined (Figure 13C and D). Whereas the combination of kanamycin and silver increased the fluoresence of the PI dye, ascribed to increased •OH production by the combined treatment, simultaneous treatment with ampicillin and silver did not lead to increased fluorescence. These results suggest that the combination of kanamycin and silver had a synergistic effect for the increased production of hydroxyl radicals and that the production of hydroxyl radical led to increased killing through disruption of bacterial membranes. Expression of *cysD* could not protect against such combined OH-mediated killing, although it protected against silver-mediated killing. However, expression of cysD did not confer any significant protection against kanamycin treatment alone. In contrast, the mechanism of antimicrobial activity of ampicillin and silver were not synergistic, although *cysD* expression can also provide some protection against ampicillin action.

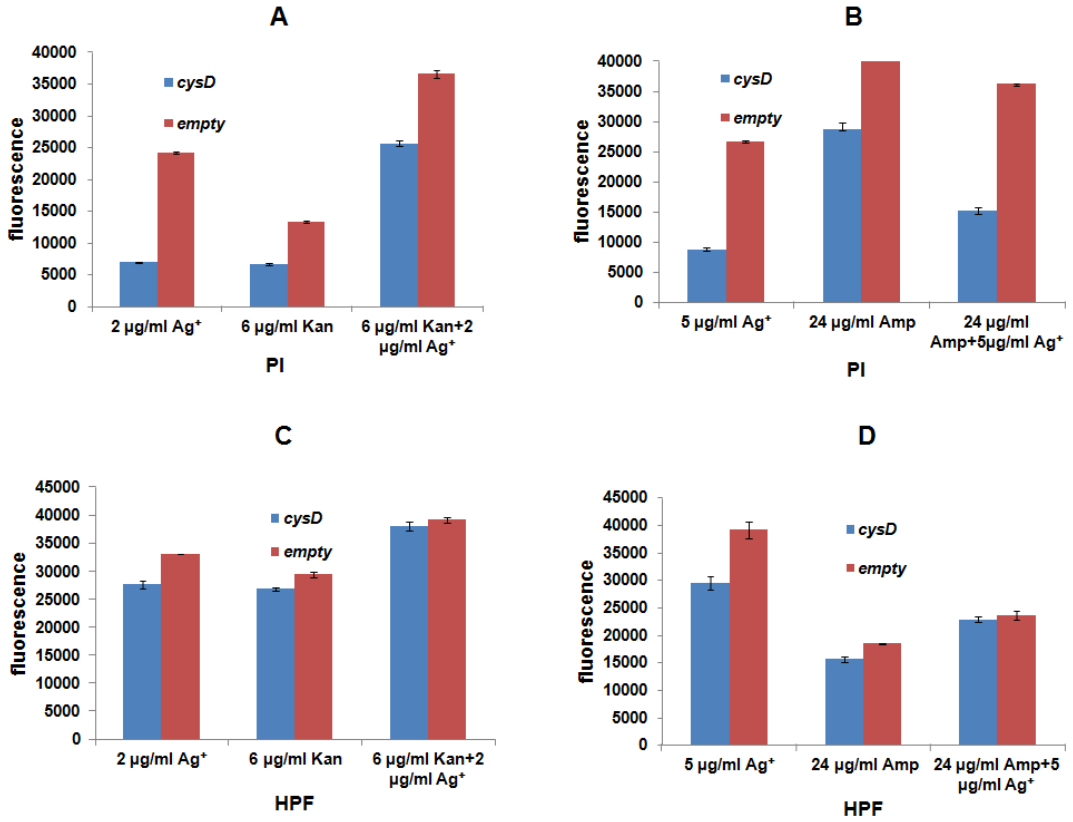


Figure 13. Detection of dead bacteria using PI and hydroxyl radicals using HPF for strains *cysD* and empty incubated in the presence of silver ion, antibiotics or in the combination of both. Detection of dead bacteria in the strains *cysD* and empty when they were incubated with (A) 2 µg/ml Ag^+, 6 µg/ml kanamycin or a combination of both and (B) 5 µg/ml Ag^+, 24 µg/ml ampicilin or a combination of both after incubation of 3.5 h. Detection of hydroxyl radicals using HPF for strains treated (C) with 2 µg/ml Ag^+, 6 µg/ml kanamycin or a combination of both and (D) 5 µg/ml Ag^+, 24 µg/ml ampicillin or a combination of both after incubation of 1 h. Amp-ampicillin, Kan-kanamycin.

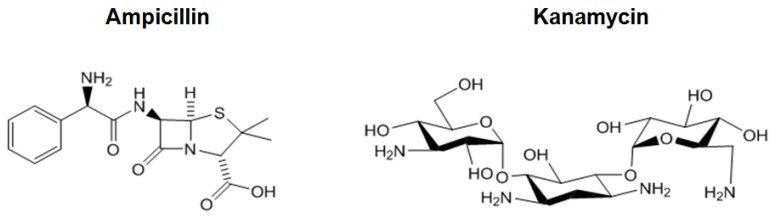


Figure 14. Structures of ampicillin and kanamycin.

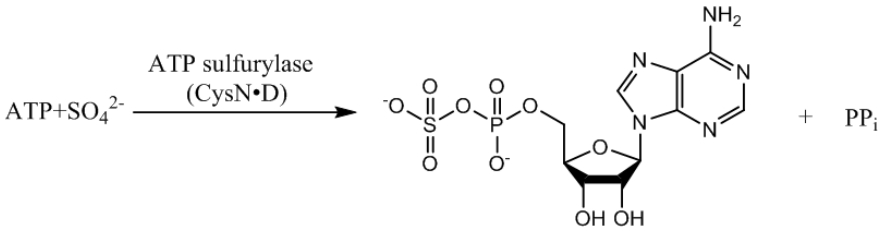


Figure 15. Generalized reaction catalyzed by ATPS enzymes.

2.3 Discussion

The antibiotic mechanisms of silver have been researched for a long time and some resistance mechanisms have been investigated, such as efflux pump system SilABC and cusCBA which can transport the silver ions to the outside of the cell, as well as detoxifying system copA,[17] silE and silF which can bind silver ion to reduce its toxicity to bacteria. By selection of ASKA plasmid genes using silver and AgNPs we obtained new anti-silver determinants, including *cysD* and *ycdB*, which endowed bacteria with cross-resistance to silver ion and AgNPs, due to the overexpression of corresponding proteins.

Although the toxicity of silver ion and AgNPs are well known and has been extensively explored, there is still controversy about how silver nanoparticles achieve their antimicrobial activity. Some believe the metallic nanoparticle itself exerts a "particle-specific" toxicity[21] while other think AgNPs achieve their antimicrobial activity by releasing silver ion.[20] Our results show that the silver resistance profile of selected ASKA strains is similar for AgNPs and for Ag ion (Figures 4 and 6). The resistance ability of ASKA strains to metallic Ag(0) depends on aeration, *i.e.* metallic silver has stronger toxicity under aerobic than anaerobic conditions. The toxicity of AgNPs is an oxygen-dependent process,[22] so strains have more resistance to metallic silver under anaerobic conditions than under aerobic conditions. We also confirmed that the large size of AgNPs has less toxicity than the small AgNPs. We ascribe this behavior to small AgNPs having larger surface, which can therefore release more silver ion. Taken together, we support the notion that the toxicity of AgNPs is exerted chiefly from silver ion, which can be released slowly from the surface of AgNPs in the presence of oxygen.

Expression of *cysD* not only gives the highest resistance ability to silver but also gives more resistance to Cd^{2+} and Cu^{+} under overexpression (Figure 7) compared to control. Ag(I) and Cu(I) possess the same outer electronic configuration, so they are closely related, and are both deemed soft

metals that preferentially combine with ligands such as sulfhydryl and nitrogen groups. Because of having the similar ligand-binding chemistries, it is not surprising that some proteins or peptides which can bind to Ag(I) also can bind to Cu(I), such as cus system.[25] Cu(I)-binding sites in proteins can often also combine to Ag(I), such as cue system.[26] The finding that *cysD*, selected for silver resistance, can also give resistance to copper supports that the antimicrobial mechanism of two kinds of metal ions is fundamentally the same. Cadmium is also a soft metal and Cd(II) can bind to sulfhydryl groups on essential proteins, interfering with important cellular functions.[27,28]

The antimicrobial activity of silver can be classified into three general mechanisms: (1) binding to thiol groups or nitrogen in proteins and disrupting their function; (2) compromising cell membranes leading to cell lysis; (3) oxidative stress from generation of reactive oxygen species. A surprising finding was that silver ion toxicity also appears to be affected by exposure to air. Our results suggest that silver can increase Fenton chemistry ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$) and induce overproduction of hydroxyl radicals in bacteria, which may be the main reason of bacterial-killing by silver ion. Ag is a redox inert metal and its thiophilic nature maybe displace Fe from Fe-S clusters, which in turn prompt the production of •OH-radical.[29] Expression of *cysD* appears to protect from to •OH-radical mediated killing. However, *cysD* expression did not confer more resistance to H_2O_2. We hypothesise that expression of *cysD* may prevent silver-mediated •OH-radical formation, although it does not appear to confer general protection against oxygen radicals.

Multidrug-resistant, Gram-negative bacteria are an important cause of nosocomial infections.[30] With the appearance of antibiotic resistant bacteria approaches to overcome such bacterial resistance must be sought. We explored the possible synergistic effects of antibiotics and silver against *E. coli* bacteria, in order to search for a more effective bactericidal method. Our experiments suggest that combination of kanamycin with silver results in a greater bactericidal effect than either of the two antibacterial reagents applied alone and that *cysD* expression, although protective against silver alone, is not protective to the combined effect of silver and kanamycin. These experiments provide solid evidence of the synergistic antibacterial effects of an antibiotic and silver, suggesting that a combination of suitable antibiotics with silver may potentiate the antimicrobial activity even against resistant strains. In marked contrast to the case of kanamycin, the combination of ampicillin and silver ion did not provide any improvement in the antibacterial activity, even for the control strain. It is possible that the lack of synergy between silver and ampicillin relates to ampicillin binding to silver ion: ampicilin is a beta-lactam antibiotic containing many active groups such as sulfur and carboxyl groups which could coordinate to silver ion, while kanamycin is an aminoglycoside bacteriocidal antibiotic (Fig 14). Such possible chelation of silver could eliminate the antibacterial activity of silver, so that only ampicillin functions as antibacterial reagent, thus leading to no increasing antibacterial activity (5 μg/ml Ag^+ and 24 μg/ml ampicillin used and the stoichiometry of metal:antibiotic is 0.717). Alternatively, it may be that ampicillin and silver mediate similar microbicidal pathways in bacteria, thus leading to a common protective mechanism by *cysD*.

Some researches have demonstrated that bactericidal antibiotics with a variety of different mechanisms of action augment ROS production within cells via the Fenton reaction.[31] So tolerance to antibiotics may rest with the ability of the cell to defend itself against ROS.[28–30] For example, the hydroxyl-radical scavenger thiourea protects the persister subpopulation,[32] the coordinated stringent response to nutrient limitation in P. aeruginosa and E. coli was shown to increase antioxidant

enzyme expression and decrease production of prooxidant molecules.[33] Intriguingly, we discovered that the anti-silver gene *cysD* also confers some resistance to the antibiotic ampicillin and kanamycin, pointing to the possible co-evolution of resistance to silver and antibiotics in bacteria. This raises the intriguing possibility that silver-resistance genes, and by extension metal-resistance genes, may contribute to multi-drug resistance in bacteria.[34]

CysD is the subunit of ATP sulfurylase and responsible for directly forming APS under control of the G protein (Figure 15).[35] ATP sulfurylase is an enzyme involved in activation and subsequent reduction of sulfate to sulfide. In order to understand the reason why *cysD* can give more resistance to silver, we first tested the overexpression amount of protein encoded by *cysD* and content of thiol group. The amount of expressed recombinant protein as measured by SDS-PAGE is very low. The low expression of CysD protein suggests that direct binding of the protein to silver is probably not its main mechanism of protection. Since CysD is involved in the cysteine anabolic pathway, we hypothesized that increased thiol-containing compounds such as glutathione could be protecting against silver toxicity. Surprisingly, the measured amount of reduced thiol groups in cell extracts of the CysD-expressing strain was similar (if anything lower) than in other strains. However, since the amount of •OH-radical formation was also lower in the CysD-expressing strain, it may be that *cysD* does increase the production of thiol-containing compounds, but these are involved in increased quenching of •OH-radicals, thus rendering them oxidized and undetectable in our assays. YcdB is reported as a member of the dye-decolorizing peroxidase (DyP) protein family with modest guaiacol peroxidase activity.[36] YcdB is a dimeric protein and contains a heme cofactor. The gene encoding YcdB protein encodes for a Tat signal. The Tat signal sequence of YcdB makes protein be translocated to the periplasm via the Tat system. In contrast to all other periplasmic hemoproteins, heme of YcdB is assembled into the protein in the cytoplasm, *i.e.* heme assembly take place before translocation.[36] YcdB might be a peroxidase, which are a large family of enzymes that typically catalyze a reaction of the form: ROOR' + electron donor (2 e^-) + 2H^+ → ROH + R'OH.[37] So the function of YcdB in the periplasm may involve a detoxification reaction under specific conditions.[25] Therefore we speculate the detoxification of silver ion of *ycdB* may arise from the peroxidase activity, which maybe directly or indirectly reduces the toxic Ag(I) to the less toxic Ag(0) and/or be involved in detoxifying reactive oxygen radicals through its redox activity. In addition, the known silver-resistance protein CueO, used here as a positive control, is also known to be translocated to the periplasm by the Tat system. These and other Tat-containing proteins are explored further in chapter 3.

Other identified proteins conferred low resistance to silver. The YabQ protein has not been explored much in *E. coli*; however, the homologue in *B. subtilis* is localized in the membrane of the forespore and is predicted to have five transmembrane domains and a signal sequence at the N-terminus region. YabQ may be a transporter for components required for synthesis of the spore cortex.[38] The YdgT protein, also known as cnu, is a largely unexplored protein of *E. coli* and a paralogue of Hha, involved in binding and regulating the activity of nucleoid-associated proteins.[39] YeaO is a hypothetical protein and there is practically no further information available in the literature or public databases. IscA, an 11 kDa member of the hesB family of proteins, binds iron and [2Fe-2S] clusters, and participates in the biosynthesis of iron-sulfur proteins.[40] The laboratory evolution of these two latter proteins is explored in chapter 4.

2.4 Conclusion

The main aim of this chapter was first to select silver resistance genes from the ASKA plasmid gene library using AgNPs and Ag ion in the absence or presence of IPTG and then to study possible mechanisms of resistance of these "anti-silver" genes. We identified several genes whose recombinant expression in *E. coli* gave increased resistance to silver ions and particles, including *cysD* and *ycdB.* Of these, *cysD* gave most resistance to silver and also gave resistance to copper and cadmium. Increased antimicrobial activity of silver in aerobic condition and fluorescence assays suggest that silver ion can induce the production of hydroxyl radical. Anti-silver genes *cysD* also endowed some resistance to ampicillin and kanamycin, suggesting that evolution of heavy-metal resistance may contribute to multiple resistance to antibiotics. However, we detected a marked synergy of antibacterial action between silver and kanamycin, even for the *cysD* strain, which had been selected for increased silver resistance. Our research using laboratory selection in a model bacterial system provides more detailed insights into mechanisms of antimicrobial silver and to the evolution multidrug resistance bacteria.

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Chapter 3 The silver-resistance activity of recombinant Tat-signal-containing genes

Introduction

In the previous chapter genes were identified whose overexpression in *E. coli* conferred increased resistance to silver. One of the genes identified codes for the enzyme YcdB, a member of the dye-decolorizing peroxidase (DyP) protein family. YcdB can catalyze the release of iron from heme and sequence analysis of YcdB revealed a twin-arginine motif within a leader sequence.[1] Proteins that are expressed with a twin-arginine motif can be exported into the periplasm after folding, by the twin-arginine translocation (Tat) pathway. Another gene encoding for a Tat-sequence, CueO, was also chosen as a positive control in the last chapter and also shown to confer detectable resistance to silver. Since two proteins encoded by genes with Tat signal sequence gave some resistance to silver, we hypothesized that other proteins expressed by the genes with a twin-arginine motif could also give more resistance to silver. This hypothesis was explored in further detail and forms the basis for this chapter.

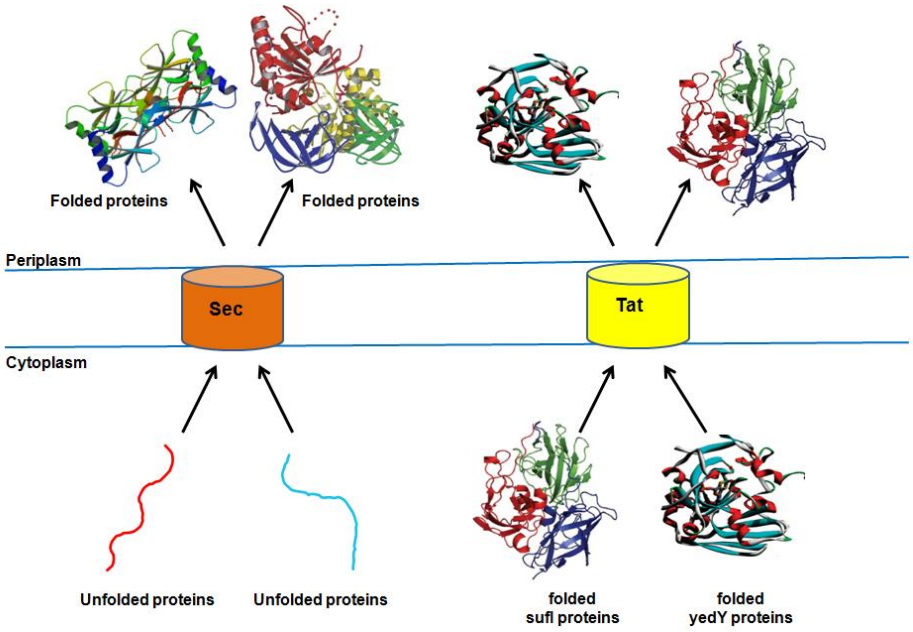

Figure 1. Schematic of routing of the Sec and Tat pathways. Unfolded periplasmic proteins are exported via Sec pathway, whereas folded conformations are exported via the Tat pathway, which transport protein with twin arginine motifs (sufI and yedY are simply shown as examples of proteins exported through the Tat pathway). ref 2

Protein translocation to the periplasm in bacteria

Prokaryotes export proteins across the cytoplasmic membrane by two parallel and complementary pathways, which are the Sec and Tat pathways (Figure 1).[2] In the Sec pathway translocation takes place through a threading mechanism, which demands the substrate translocated to be in an outstretched conformation (unfolded conformation). In contrast, the Tat (twin-arginine translocation)

pathway is devoted to the transport of folded proteins, *i.e.* only proteins that can attain a folded state in the cytoplasm are competent for export via Tat pathway. Proteins are targeted to the Tat pathway by N-terminal signal peptides. These signal peptides possess continuous, essentially fixed, arginine residues within an S–R–R–x–F–L–K consensus motif, named "twin arginine" (RR) signal peptides.[3] Tat-targeted proteins are synthesised as precursors with N-terminal signal peptides. Twin-arginine signal peptides possess a tripartite structure consisting of a polar N-terminal region (n-region), a moderately hydrophobic region (h-region), and a C-terminal region (c-region) in which a protease cleavage site is often contained (Figure 2).[4] The twin-arginine motif is always positioned at a consensus motif at the n-region–h-region boundary.[4] Efficient targeting of passenger proteins in Tat pathways is mainly dependent on the twin arginines of the signal peptide. In Gram-negative bacteria and plant chloroplasts, three membrane proteins, which are termed TatA, TatB and TatC in bacteria, consist of Tat machineries. TatA includes a single transmembrane domain and a cytosolically located amphipathic helix followed by an unstructured C-terminus. The homologous TatB subunit that is yet functionally distinct has the same predicted structure as TatA. TatC was shown to be involved in recognizing twin-arginine signal peptides and mediates the transmembrane insertion of a twin-arginine signal sequence, thereby translocating the signal sequence cleavage site across the bilayer. On the other hand TatC has six predicted transmembrane helices in which the N- and C-termini of the protein face the cytoplasm (Figure 3).[4] The TatBC complex is related to binding RR-signal peptides as involved by multiple experimental strategies.[5,6] The homo-oligomeric TatA complex are arranged as rings to form pore-like structures, in which the protein is translocated acrossing through this channel formed by TatA protomers.

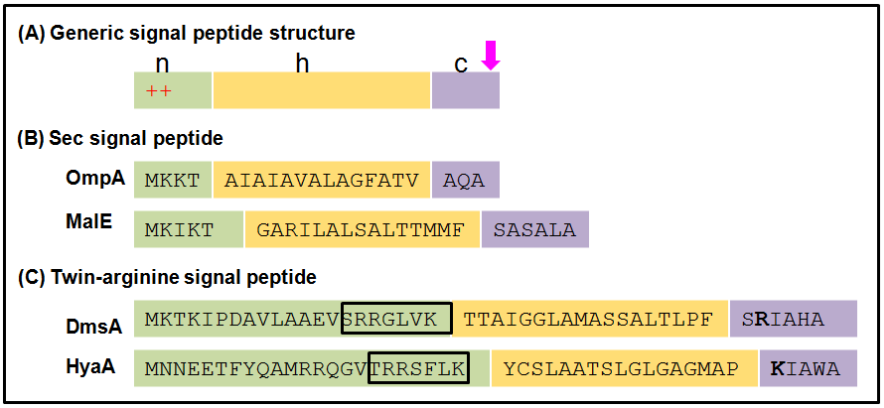

Figure 2. The tripartite structure of Sec and Tat signal peptides. (a) The generic tripartite structure of both Sec and Tat signal peptides. Both types of signal peptide encompass an N-terminal positively charged region of variable length (n-region, shaded green), followed by a hydrophobic h-region (shaded orange) normally containing 15–20 amino acid residues, and a more polar C-region (shaded purple) containing the A×A (or an acceptable variation thereof) signal peptidase-I recognition sequence. (b) Two examples of extensively characterized Sec signal peptides from Escherichia coli. Other than the signal peptidase cleavage site in the c-region, the outer membrane protein A (OmpA) and periplasmic maltose-binding-protein (MalE) signals share no obvious amino acid conservation. (c) Some representative twin-arginine signal peptides. The conserved residues that contribute to the twin-arginine motif are boxed in each case and always lie close to the n-region–c-region boundary. The positions of highly conserved basic amino acids within the c-regions of Tat signal peptides are shown in bold. This feature is often referred to as the 'Sec-avoidance motif '.[7]

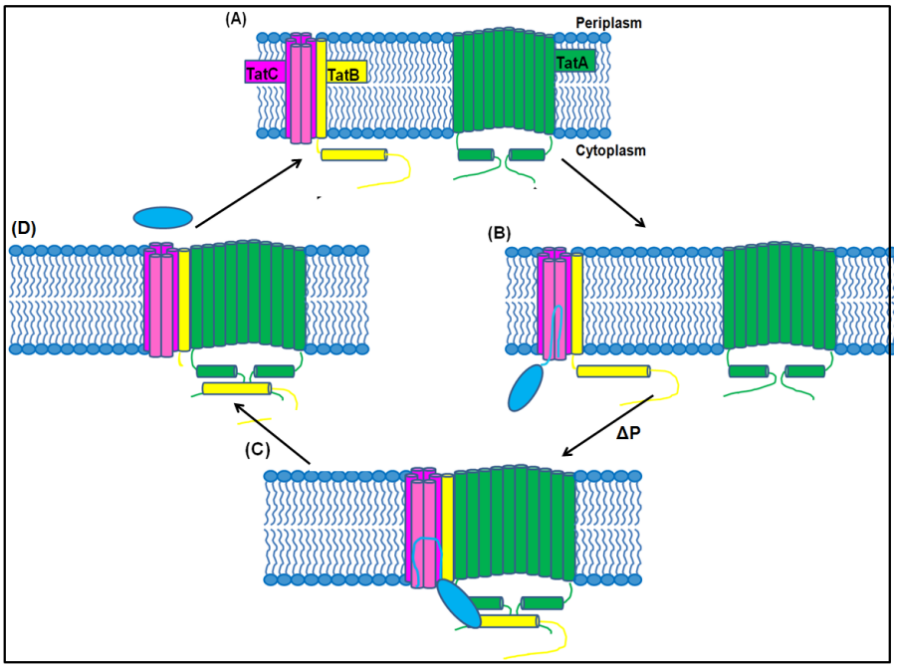

Figure 3. A model for the Tat transport cycle. This model is based on complementary biochemical studies on the *Escherichia coli* and plant thylakoid systems. For clarity, arbitrary numbers of TatA protomers (green) and single copies of the TatB (yellow) and TatC (red) proteins are shown. Under resting (non-translocating) conditions, TatA and TatBC form separate high-molecular-mass complexes within the membrane. (a) A depiction of the *E. coli* inner membrane is shown with the relative positions of the periplasm and cell cytoplasm shown. (b) The cycle is initiated when the twin-arginine signal peptide of a Tat substrate protein (blue) binds the TatBC complex in the membrane. The twin-arginine motif is recognized directly by TatC.[8,9] (c) In a proton-motive force (**Δ**p)-dependent manner, the TatA complex then associates with the substrate-bound TatBC module. (d) The TatABC complex is now fully assembled, and the substrate protein is translocated across the membrane through a channel formed by multiple TatA protomers. Protein transport is probably driven by the transport of protons across the membrane. Following transport, the TatA and TatBC complexes dissociate and return to the resting state (a).[10,11] Adapted from publications ref 4.

Transport by the Tat pathway is energized solely by the membrane potential proton-motive force[12] and the costs for translocation appear to be very high, which may be the reason why many organisms prefer the less energy-consuming translocation of unfolded proteins via the Sec apparatus. The most complete Tat substrate analyses have been reported from *Escherichia coli*.[4] Many Tat substrates from *E. coli* need to be translocated in a folded conformation because they are heterodimers or because they are co-assembled with a cofactor in the cytoplasm.

The *Escherichia coli* genome encodes at least 27 putative signal peptides containing a twin arginine motif characteristic of proteins exported via the twin arginine translocation pathway (Table 1).[4] Here, we first tested anti-silver ability of Tat genes from *E. coli* and selected those having high resistance ability upon recombinant expression for further exploration. Five Tat-containing genes were identified that gave more resistance to silver than the previously-characterised *ycdB* and *cueO* genes. Further we functionally tested these 7 Tat-containing genes, which gave more resistance to silver compared to empty-plasmid controls, using recombinant constructs in which we deleted either the

Tat signal peptide, the His tag, the insert sequence or combinations thereof in order to research their effect of these sequences on anti-silver ability of bacteria.

Table 1. A complete list of known and predicted Escherichia coli Tat substrates

Protein	Physiological role	Cofactors	Co-exported partner	Signal chaperone
HyaA	Hydrogen oxidation	3×Fe-S clusters	HyaB	HyaE
HybO	Hydrogen oxidation	3×Fe-S clusters	HybC	HybE
HybA	Hydrogen oxidation	4×Fe-S clusters[b]	Unknown	Unknown
NapG	Nitrate reduction	4×Fe-S clusters[b]	Unknown	Unknown
NrfC	Nitrite reduction	4×Fe-S clusters[b]	Unknown	Unknown
YagT	Unknown	2×Fe-S clusters[b]	YagR[b], YagS[b]	YagQ[b]
YdhX	Unknown	4×Fe-S clusters[b]	Unknown	Unknown
TorA	TMAO reduction	MGD	None	TorD
TorZ	TMAO reduction	MGD	None	YcdY[b]
NapA	Nitrate reduction	MGD, 1×Fe-S cluster	None	NapD[b]
DmsA	DMSO reduction	MGD, 1×Fe-S cluster	DmsB	DmsD
YnfE	DMSO reduction	MGD, 1×Fe-S cluster[b]	YnfG[b]	DmsD[b]
YnfF	DMSO reduction	MGD, 1×Fe-S cluster[b]	YnfG[b]	DmsD[b]
FdnG	Formate oxidation	MGD, 1×Fe-S cluster	FdnH	FdhD[b], FdhE[b]
FdoG	Formate oxidation	MGD, 1×Fe-S cluster[b]	FdoH[b]	FdhD[b], FdhE[b]
YedY	TMAO/DMSO reduction	MPT	None	Unknown
CueO	Copper homeostasis	4×Cu ions	None	Unknown
SufI	Possibly cell division	None	Unknown	Unknown
YahJ	Unknown	1×Fe ion[b]	Unknown	Unknown
WcaM	Colanic acid biosynthesis	Unknown	Unknown	Unknown
MdoD	Glucans biosynthesis	Unknown	Unknown	Unknown
YcdB	Unknown	Unknown	Unknown	Unknown
YcdO	Unknown	Unknown	Unknown	Unknown
YaeI	Possible phosphodiesterase	Unknown	Unknown	Unknown
AmiA	Cell wall amidase	None	Unknown	Unknown
AmiC	Cell wall amidase	None	Unknown	Unknown
FhuD	Ferrichrome binding	None	Unknown	Unknown
YcbK	Unknown	Unknown	Unknown	Unknown

[a]The *E. coli* K-12 genome encodes 27 polypeptides known or predicted to bear N-terminal twin-arginine signal peptides, which represents ～ 6% of cell-envelope proteins (currently estimated total of 452 in E. coli K-12; http://www.cf.ac.uk/biosi/staff/ehrmann/tools/ecce/ecce.htm). Many of these proteins bind redox cofactors, such as iron-sulphur clusters (Fe-S), molybdopterin guanine dinucleotide (MGD) or molybdopterin (MPT) (Figure 4). Some are co-exported with a partner protein that contains no signal peptide itself, and many require that activity of signal peptide-binding 'proofreading' chaperones to coordinate the assembly and export processes. [b]Predictions inferred by homology, genetic linkage or sequence analysis; see Berks et al.[5] and Turneret al.[13] for recent reviews.

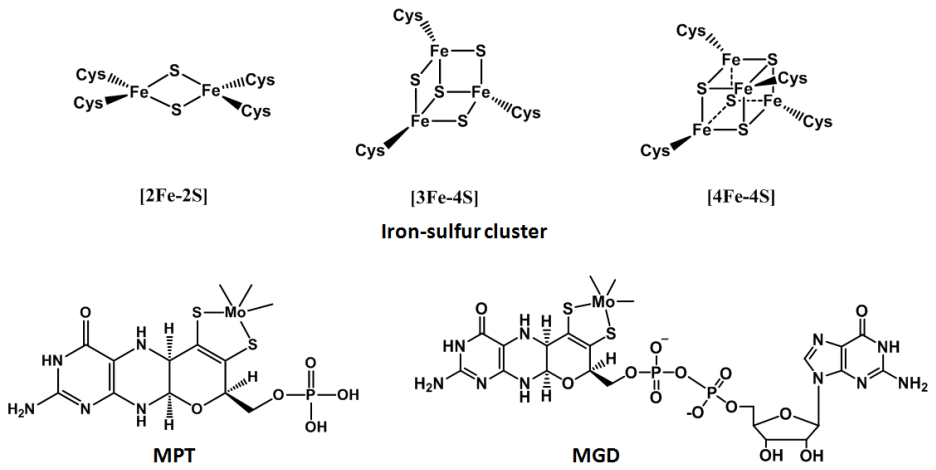

Figure 4. Structure of the iron-sulfur clusters and molybdopterin cofactors found in Tat-containing proteins of *E. coli*. ref 14

3.1 Materials and methods

Table 2. Overview of recombinant gene constructs explored. Blue titles display the part deleted from genes. Black bold is the name of recombinant genes. The symbol Δ denotes the deleted fragment. For deletions, the indicated pairwise forward and reverse primers were used for whole-plasmid PCR.

Deletion of TAT signal sequence (ΔT-)			Deletion of His tag and TAT (ΔTΔH-)			Deletion of His tag (ΔH-)			Template
name	Forward primer	Reverse primer	name	name	Forward primer	Reverse primer	name	name	Forward primer
ΔT-ycdB	ycdB+	ASKA_delTAT-	**ΔTΔH-ycdB**	**ΔT-ycdB**	ycdB+	ASKA_delTAT-	**ΔTΔH-ycdB**	**ΔT-ycdB**	ycdB+
ΔT-cueO	cueO+	ASKA_delTAT-	**ΔTΔH-cueO**	**ΔT-cueO**	cueO+	ASKA_delTAT-	**ΔTΔH-cueO**	**ΔT-cueO**	cueO+
ΔT-hybA	hybA +	ASKA_delTAT-	**ΔTΔH-hybA**	**ΔT-hybA**	hybA +	ASKA_delTAT-	**ΔTΔH-hybA**	**ΔT-hybA**	hybA +
ΔT-torA	torA +	ASKA_delTAT-	**ΔTΔH-torA**	**ΔT-torA**	torA +	ASKA_delTAT-	**ΔTΔH-torA**	**ΔT-torA**	torA +
ΔT-yedY	yedY +	ASKA_delTAT-	**ΔTΔH-yedY**	**ΔT-yedY**	yedY +	ASKA_delTAT-	**ΔTΔH-yedY**	**ΔT-yedY**	yedY +
ΔT-sufI	sufI+	ASKA_delTAT-	**ΔTΔH-sufI**	**ΔT-sufI**	sufI+	ASKA_delTAT-	**ΔTΔH-sufI**	**ΔT-sufI**	sufI+
ΔT-ycdO	ycdO+	ASKA_delTAT-	**ΔTΔH-ycdO**	**ΔT-ycdO**	ycdO+	ASKA_delTAT-	**ΔTΔH-ycdO**	**ΔT-ycdO**	ycdO+

Deletion of gene (express only TAT and His) (ΔG-)			Template	Deletion of His and gene (express only TAT). Two step-construction.						
name	Reverse primer	Forward primer		Name	step1 delete His		template	step2 delete genes		template
					Reverse primer	Forward primer		Reverse primer	Forward primer	
ΔG-ycdB	ycdB-	ASKA_delgene+	ycdB	**ΔGΔH-ycdB**	ASKA_delHis-	ASKA_delHis+	ycdB	ycdB-	ASKA_delgene+	ΔH-ycdB
ΔG-cueO	cueO-	ASKA_delgene+	cueO	**ΔGΔH-cueO**	ASKA_delHis-	ASKA_delHis+	cueO	cueO-	ASKA_delgene+	ΔH-cueO
ΔG-torA	torA-	ASKA_delgene+	torA	**ΔGΔH-torA**	ASKA_delHis-	ASKA_delHis+	torA	torA-	ASKA_delgene+	ΔH-torA

Table 3. Primer sequences used for different candidates Tat genes in the recombinant of genes.

Name of primer	Sequence of primer	Name of primer	Sequence of primer
ycdB+	5' P CAA AAA ACG CAA AGT GCG CCG GGT AC 3'	ASKA_delTAT-	5' P CGG ATC CGT ATG GTG ATG GTG ATG 3'
cueO+	5' P GCA GAA CGC CCA ACG TTA CC 3'	ASKA_delHis-	5' P GTG AGA TCC TCT CAT AGT TAA TTT CTC CTC 3'
hybA+	5' P GCT GAA AAC CGC CCG CCA ATT C 3'	ASKA_delHis+	5' P aAT ACG GAT CCG GCC CTG AG 3'
torA +	5' P CAA GCG GCG ACT GAC GCT GTC ATC TCG AAA G 3'	ycdB-	5' P TTA TGC ATG AGC GAC CGG ACA AC 3'
yedY+	5' P GAT CTG CTT AGC TGG TTT AAA GG 3'	cueO-	5'P TTA CGC AAA TAC TGC GCG GCT C 3'
sufI+	5' P GCA GGA CAA CAG CAA CCG CTA CCC GTT C 3'	torA-	5'P TTA CGC AGT CGC ACG TCG CGG CGT TAA C 3'
ycdO+	5' P GCT GAT GTG CCG CAG GTC AAA GTG 3'	ASKA_delgene+	5'P AGG AGA AGA ACT TTT CAC TGG AGT TG 3'

The *E. coli* strain used in this work was XL1-Blue. LB-medium was used as a rich medium. Tetracycline, chloramphenicol and IPTG were used at final concentrations of 5, 34 µg/ml and 1mM, respectively, when added to either liquid or solid medium. Electrocompetent cells and chemical competent cells of *E. coli* were prepared by standard protocols. Agar (used at 1.5%) and agarose (used at 1%) were purchased from Invitrogen. Turbo Pfu polymerase and other restriction enzymes are from New England Biolabs, unless specified. All other chemicals were purchased from Applichem, unless otherwise specified. Primers were synthesized from Microsynth, Switzerland and the gene sequencing was from Starseq, Germany. *E. coli* XL1-Blue strains were routinely grown at 37 °C with IPTG in Luria-Bertani (LB) medium before adding silver ion supplied as silver nitrate or on LB plates. When adding silver ion, the cultures were incubated at 25°C. Metallic Ag(0) stabilized in gelatine was generously provided by Nanotrade.cz.

3.1.1 Selection of ASKA Tat signal genes with high resistance to silver ion.

All plasmids containing genes with Tat signal sequences listed in Table 1 were isolated from the ASKA library and transformed into XL1-Blue cells by standard chemical transformation. Single colonies from every ASKA Tat strain was tested for resistance to silver according to the experiment process 2.1.4. Briefly, the cultures containing preculture, chloramphenicol and tetracycline in the presence of IPTG were incubated to OD_{600} 0.4 at 37 °C. Silver nitrate (6 µg/ml for silver) was added at OD_{600} 0.4 and the cultures containing the ASKA plasmids were incubated for 7 h at 25°C. The samples were taken at 4 and 7 h followed by dropping 5 µl of different diluted culture to big petri plates. These plates were incubated for 24 h at 37 °C for counting of colony forming units (cfu). By Five genes were found to confer more resistance to silver than the empty plasmid: *hybA*, *torA*, *yedY*, *sufI* and *ycdO*.

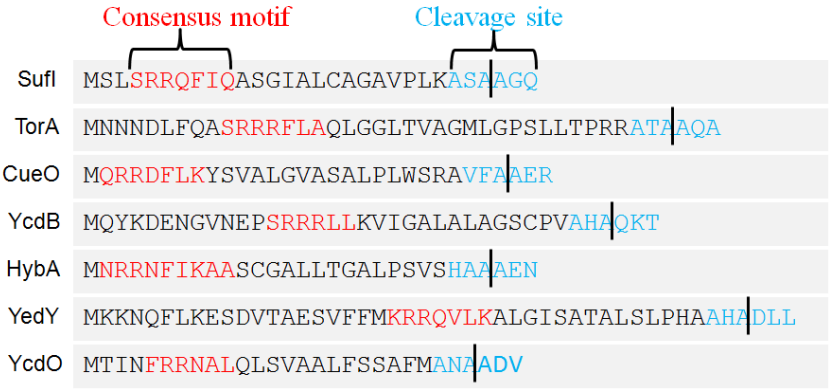

Figure 5. Sequences of the Tat signal peptides of selected proteins tested. The consensus motif is shown in red and the cleavage site in blue. The vertical black line indicates the site of cleavage by signal peptidase I. ref 2,13

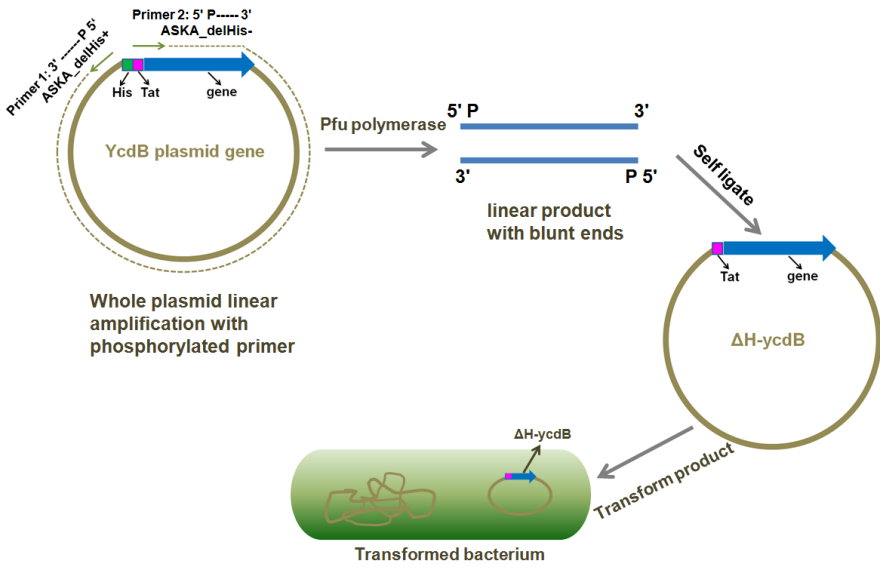

Figure 6. General strategy for deleting sequence of interest in recombinant genes (illustrated here is deletion of His tag of ycdB, as a representative example).

3.1.2 Recombinant constructs of selected genes with Tat signal sequences

To research effect of the Tat signal sequence and His tag on the anti-silver ability of genes, we chose to delete Tat signal peptides, both Tat signal peptides & His tag, or His tag alone for 7 selected genes: *cueO, ycdB, hybA, torA, yedY, sufI* and *ycdO* (Figure 5 shows the Tat signal sequences of these 7 genes). As a control and to research the effect of these genes on their anti-silver ability, we also deleted the gene-insert or both the gene-insert and His-tag of *ycdB* (originally selected from whole ASKA library for silver resistance in chapter 2); *cueO* (positive control) and *torA* (which gave the strongest resistance to silver of the 5 Tat genes selected), *i.e.* only keeping Tat or Tat and His. All recombinant genes and the pairwise primers used in the PCR reaction are listed in Table 2. The ASKA plasmids were modified by PCR by using primers listed on Table 3. Initially, the corresponding ASKA plasmids *hybA, torA, yedY, sufI, ycdO, cueO* and *ycdB* were used as a template for each PCR reaction. For example, ΔH-*ycdB* (deleting of His tag sequence) was constructed from wildtype *ycdB*. The His-tag sequence coding His region was deleted by using the forward primer ASKA_delHis- (5' P GTG AGA TCC TCT CAT AGT TAA TTT CTC CTC 3') and the reverse primer ASKA_delHis+ (5' P aAT ACG GAT CCG GCC CTG AG 3') in the PCR. For all PCR the following conditions were used: hot start PCR, with initial 95°C for 5 minutes, then 40 cycles of 95°C/ 1 minute, 55°C/ 1 minute and 68°C/ 10 minutes, with a final extension of 30 minutes at 68°C. Pfu Turbo DNA polymerase ($T_{elongation}$ 68°C) was used in order to avoid the errors of the Taq DNA polymerase, especially considering that the whole plasmid was amplified. Table 4 shows the PCR mixture prepared. The products of the PCR were checked on 1% agarose and the original template plasmid was removed by adding 2 µl of Dpn1 restriction enzyme (20.000 U/ml) to 45 µl of PCR products and incubating at 37 °C for 2.5 h. After this step the mixture was cleaned up by NucleoSpin® Gel and PCR Clean-up kit. One µl of Roche T4 ligase (400.000 U/ml) was added to the DNA (26 µl) premixed with T4 ligase buffer 10X (3 µl) for overnight ligation at 16°C (in a PCR machine). A scheme to illustrate the PCR strategy for deleting these sequences is shown in Figure 6 using ΔH-*ycdB* as an example. Recombinant genes were modified by PCR using identical protocols

with the corresponding templates and primer-pairs. The recombinant plasmids were checked by sequencing the insert using ASKA forward primer (5' GGC GTA TCA CGA GGC CCT TTC GTC TTC ACC TC 3'). A cartoon structure of these recombinant genes by deleting His, Tat, gene or any both are shown in Figure 7.

Table 4. Components and concentrations of the PCR reactions.

	Component	Quantity (µl)	Final concentration	Notes on stock solutions
1	ddH_2O	25.5		
2	pfu buffer	5.0	1X	10X
3	dNTPs	2.5	0.5 mM	10 mM
4	DNA template (plasmid)	1.0	0.01 ~0.5 µg	
5	forward primer	6.0	0.3 µM	2.5 µM
6	reverse primer	6.0	0.3 µM	2.5 µM
7	DMSO	2.5	4%	Additive to improve selective hybridisation of primers
8	pfu polymerase	1.5		Added at the end

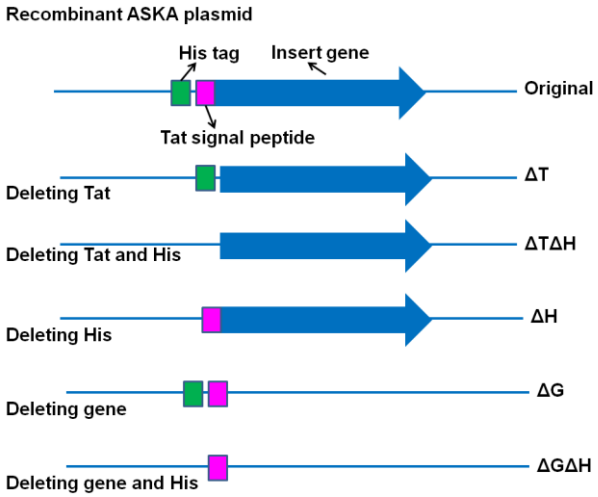

Figure 7. Cartoon representing the structure of the recombinant ASKA genes with Tat signal sequence. The nomenclature used is specified on the right for each construct and the deleted part is labeled under every construct.

3.1.3 Testing anti-silver ability of all recombinant genes

We compared the anti-silver ability of each set of the 27 modified Tat-sequence containing plasmids with CysD as non-Tat containing positive control, by transforming the plasmids separately and picking single colonies from every strain according to the methods described in section 2.1.4. Briefly the cultures containing preculture, chloramphenicol and tetracycline in the presence of IPTG were incubated to OD_{600} 0.4 at 37 °C, whereupon 6 µg/ml silver ion was added and incubated for 7 h at 25°C. Samples were taken at 3.5 and 7 h followed by spreading 100 µl of 100×diluted culture to each plate. These plates were incubated for 24 h at 37 °C for colony-counting.

3.1.4 Analysis of periplasmic and spheroplastic recombinant proteins expressed in the presence of IPTG

In order to detect the localisation of proteins encoded by Tat genes or their derivatives, enriched periplasmic and spheroplastic fractions were prepared with a Peripreps^{TM} Periplasting Kit. One milliliter (up to 2 OD_{600}) of a fresh bacterial cell culture was taken and centrifugated in a microcentrifuge. Then the pellet was thoroughly resuspended in 50 µl of PeriPreps Periplasting Buffer and incubated for 5 minutes at room temperature; after, 50 µl of purified water at 4°C was added and mixed, incubating this mixture for 5 minutes on ice. Further, the lysed cells were pelleted by centrifugation in a microcentrifuge for 2 minutes at room temperature followed by transfer of the supernatant containing the periplasmic fraction to a clean tube. The pellet was resuspended in 100 µl of PeriPreps Lysis Buffer with 0.2 mg/ml DNAsel; 1 µl of 1.0 M $MgCl_2$ was added and mixed by inversion. This lysis buffer was incubated for 5 minutes at room temperature and cellular debris was precipitated by centrifugation in a microcentrifuge for 5 minutes at room temperature. Finally the supernatant containing the spheroplastic fraction was transferred to a clean tube and 2 µl of 500 mM EDTA was added to chelate the magnesium. The periplasmic and spheroplastic fractions were analyzed by SDS-PAGE.

3.1.5 Comparison of silver-resistance activity of Tat genes under anaerobic and aerobic conditions

To explore the effect of oxygen on the anti-silver ability of Tat genes *ycdB*, *cueO* and *torA*, we investigated the effect of incubation of cells under anaerobic and aerobic conditions. First we transferred the plasmids containing three genes into XL1Blue cells and picked single colonies from every strain. The cultures inoculated were cultured at 37 °C with chloramphenicol and tetracycline in the presence of IPTG (1 mM). When OD_{600} of cultures reached 0.4, the culture was split in two. In one set, nitrogen was charged and 55 µg/ml metallic silver(0) added to one set of the pair. The other set of each culture was incubated in air in presence of the same concentration of metallic silver(0). All cultures, with and without oxygen were incubated for 8 h at 25 °C. Samples were taken at defined time intervals followed by spreading 100 µl of 100×diluted culture to each plate and incubating the plates for 24 h at 37 °C for colony counting.

3.2 Results

3.2.1 Selection of Tat genes with high resistance to silver ion

An initial exploration of the growth of 27 strains upon IPTG induction, each strain containing a different ASKA plasmid coding for a gene with a Tat-signal, revealed that overexpression was apparently toxic in 5 different strains, as shown by lack of growth after 3 hr incubation. Therefore, only 22 different ASKA strains were tested further with 6 µg/ml silver ion. Amongst these strains, those expressing *hybA*, *torA*, *yedY*, *sufI* and *ycdO* showed most resistance to silver ion, as revealed by increased numbers of surviving colony-forming units (cfu) after 4 and 7 h of incubation with silver ion (Figure 8).

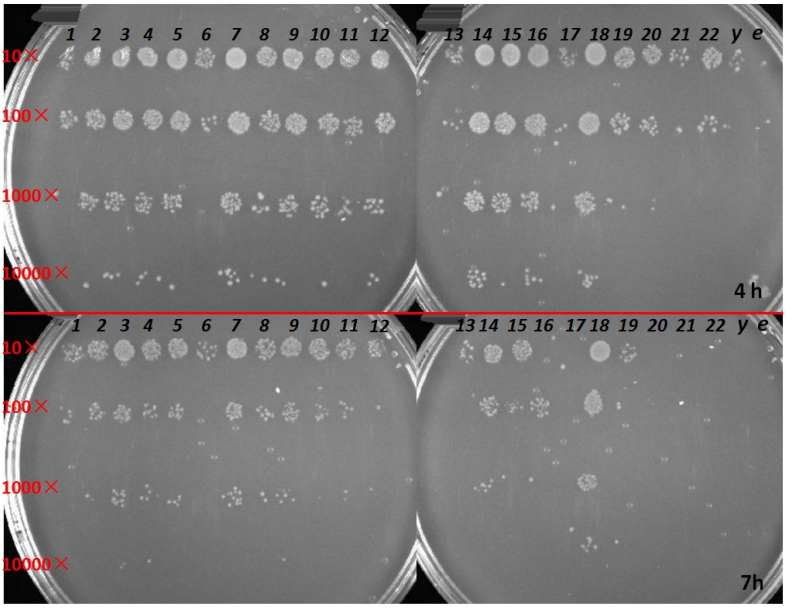

Figure 8. The resistance ability of strains with Tat genes to 6 μg/ml Ag^{+} after 4 and 7 h of incubation. The right red number is the times diluted for the culture and black number is the different Tat genes. 3-*E. coli*/*hybA*, 7-*E. coli*/*torA*, 14-*E. coli*/*yedY*, 15-*E. coli*/*sufI*, 18-*E. coli*/*ycdO*, y-*ycdB*, e-empty.

3.2.2 Confirmation of silver-resistance conferred by *cysD* and Tat genes

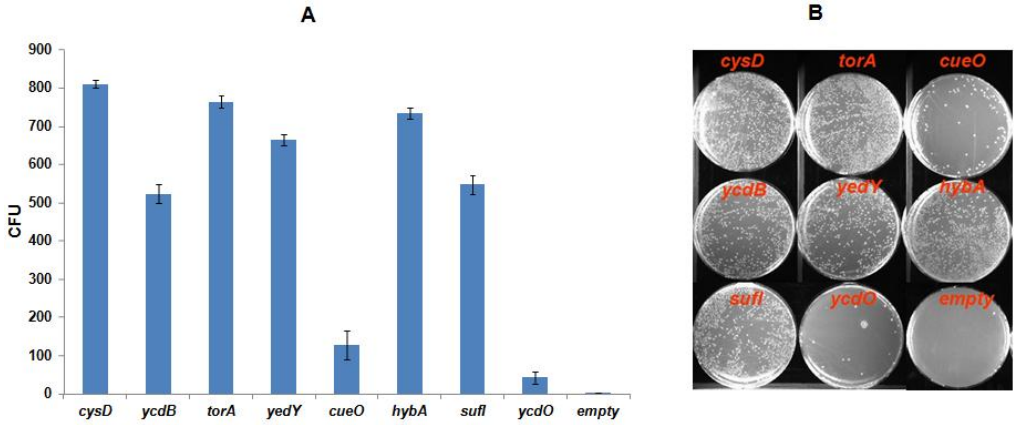

Figure 9. The resistance of strains with *cysD* and Tat genes to 6 μg/ml Ag^{+} after 7 h of incubation. (A) Bar graph of colony forming units (cfu) of all strains. (B) representative corresponding Petri dishes. Error bars represent means SD (standard deviation) from three replicates.

The silver-resistance ability of the strains expressing tat-containing proteins was confirmed by comparison with *cysD* and *ycdB* controls, identified in the last chapter. Although after 3.5 h of incubation with silver ion all strains exhibited similar resistance (data not shown), clear differences in resistance were revealed at 7 h incubation with silver ion (Figure 9). Strains harbouring *cysD*, *torA*, *yedY* and *hybA* had more survivors upon incubation with silver ion than *ycdB*, which in turn had more survivors than *cueO* and *ycdO*. These latter strains, however, still gave more survivors than the

host harbouring an "empty" plasmid, used as negative control. These results confirmed the strong anti-silver ability of the selected gene *cysD* and, in addition, demonstrated that expression of some Tat genes also endow more resistance to silver.

3.2.3 Testing of anti-silver ability of all recombined genes

Many different TAT proteins conferred resistance to silver, which led us to ask whether Tat-mediated export of the His-tag, which is a metal-binding motif and is expressed in all these constructs, could be involved in protection against silver. To answer this question we deleted the His-tag (ΔH-) of the 7 selected Tat genes and tested their silver-resistance (Figure 10A). The cfu of the original plasmids and those lacking the His-tag were similar for *sufI*, *hybA*, *ycdO*, *torA* and *ycdB*, indicating that His tag has little effect for these 5 Tat proteins. Deletion of the His tag of *yedY* and *cueO* led to increase in cfu, suggesting that the His-tag of both *yedY* and *cueO* hinders resistance to silver ion. These findings results indicated that the deletion of the his-tag in all the constructs tested either had little effect on resistance or actually increased resistance in the case of *yedY* and *cueO*.

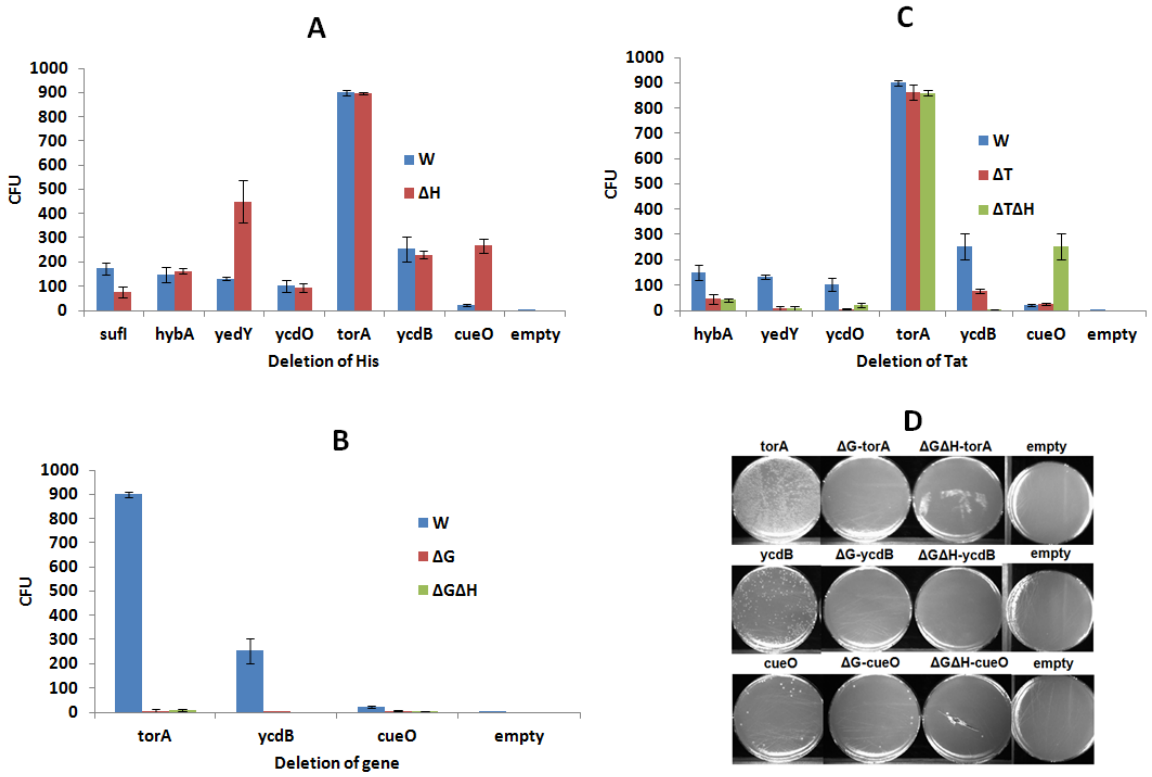

Figure 10. The resistance of recombinant strains to 6 μg/ml Ag^{+} after 7 h of incubation. Bar graph of (A) deleting of His-tag, (B) deleting of gene, (C) deleting of Tat signal sequence. (D) Petri dishes of original and recombinant genes of *torA*, *ycdB* and *cueO*. Error bars show standard deviations from three replicates. W-wildtype, ΔH-without His tag, ΔG-without gene, ΔHΔG -without His tag & gene, ΔT-without Tat sequence, ΔHΔT-without His tag & Tat sequence.

As controls, expression of just the Tat sequence alone or Tat sequence together with the his-tags of three selected genes (*torA*, *ycdB*, *cueO*) led to the elimination of resistance to silver and similar resistance to the negative control strain containing the "empty" plasmid (Figure 10B and 10D).

These results support the notion that the silver resistance of these genes is conferred by the specific proteins expressed by the Tat-containing genes. We conclude, therefore, that resistance is related to gene function and not to the His-tag or Tat-sequences, either alone or in combination.

Finally, we explored the effect of deletion of Tat alone or together with His-tag, *i.e.* expression of "mature" protein alone, without signal sequences, for *hybA*, *yedY*, *ycdO*, *torA*, *ycdB* and *cueO* (the overexpressions of ΔT-*sufI* and ΔTΔH-*sufI* are toxic to bacteria, so bacteria almost did not grow in the presence of IPTG). In most cases, there was a reduction of resistance as shown in Figure 10C: in particular deletion of Tat of *hybA*, *yedY* and *ycdO* decreased the number of cfu, most especially for *yedY* compared to corresponding wildtype. It is noteworthy that although deletion of Tat increased sensitivity of these strains to silver, their resistance was still above background, as determined by comparison with the "empty plasmid" control. Surprisingly, in the case of *torA* and *cueO*, deletion of Tat had little effect on resistance; we hypothesise that these proteins may be co-transported to the periplasm in a complex with other Tat-exported proteins.[17] For *cueO* deleting both Tat & His markedly increased silver-resistance, which suggests that the His tag interferes with expression or function of the gene-product (this trend is in line with the finding of increased silver resistance of ΔH-*cueO*).

3.2.4 Analysis of periplasmic and spheroplastic proteins encoded by Tat

Proteins encoded by genes with Tat signal peptides can be transported by Tat pathway from cytoplasm to periplasm. To explore the effect of TAT deletion on protein expression and localisation, we carried out SDS-PAGE analyses on periplasmic and spheroplasmic fractions of TorA, CueO, YedY, and YcdB according to The PeriPreps™ Periplasting Kit (Figure 11). SDS–PAGE analysis of Periplasmic and Spheroplastic proteins revealed different protein patterns, suggesting successful fraction-enrichment.

For CueO, very strong overexpression was found in both fractions. CueO (and ΔH-CueO) showed two overexpressed species, especially evident in spheroplastic fractions, which corresponded to peptide-containing (58.404 KDa) and mature CueO (55.474 KDa). The presence of mature CueO existing in the spheroplastic part may simply be the result of translocon saturation: signal sequences of accumulating Tat substrates are often found to be sensitive to proteases, resulting in digestion of the signal sequence while the folded mature part of the protein remains intact. Accordingly, proteolytic degradation up to the mature folded domain is often cytoplasmically observed with recombinant Tat substrates *in vivo* when translocation becomes limiting, as described for YcdB.[15] For wildtype CueO, only an overexpressed species corresponding to mature CueO was found in the periplasm, but no pre-CueO was observed, which indicates purified periplasmic fractions, in which the 58.404 kDa precursor form of CueO of the cytoplasm is not present, but only the processed mature form with size 55.474 kDa. The protein level of CueO in the periplasm was relatively poor. This is possibly because the Tat system is slow and becomes rapidly saturated.[16] Expression of ΔH-*cueO* in the periplasm (mature protein only) was much weaker and only a weak faint band seen, indicating the His tag may play some role in the translocation of pre CueO from cytoplasm to periplasm. For ΔT-*cueO* and ΔTΔH-*cueO*, even with TAT deleted, abundant protein was found in the periplasm. This finding suggests that CueO can be exported to the periplasm independent of its own Tat sequence, perhaps by co-translocation with other Tat-containing proteins. We believe that

the presence of ΔT-CueO and ΔTΔH-CueO in periplasmic fractions is not just due to an artefact of the preparation such as spheroplasmic contamination, because in the wildtype control (and his-tag-deleted fraction) only the spheroplasmic fraction contained a larger species corresponding to the "non-processed" form with signal peptide. Comparing ΔT-CueO and ΔTΔH-CueO, the amount of both proteins was similar in spheroplasmic fractions, but recombinant ΔTΔH-CueO protein was less abundant than ΔT-CueO in the periplasm, also indicating that deletion of His-tag appears to affect expression and export, although such deletion leads to higher resistance. These results support the notion that the His-tag appears to interfere with the function and/or silver resistance of *cueO*.

For YedY, recombinant protein was weakly expressed (in the spheroplastic fraction) and was not visible in the periplasm, suggesting that significant silver resistance was achieved with low protein levels. As for *cueO*, even with the deletion of Tat, the TorA protein was found abundantly in the periplasm (as well as in the spheroplasmic fraction). It is noteworthy that Tat deletion of *torA* led to higher expression both in spheroplastic and periplasmic fractions. However, visibly increased expression of recombinant protein in ΔT-*torA* did not correlate with increased resistance to silver (Figure 10C); we speculate that the presence of a Tat sequence or slow expression may be important for assembling a functional protein, for example aiding in cofactor binding. Similarly, Tat deletion of *ycdB* also led to higher expression and, as for TorA, even with the deletion of Tat, the recombinant protein was also found abundantly in the periplasm (as well as in the spheroplasmic fraction). For all proteins explored, deleting the Tat sequence gave much greater expression (Figure 11), but this did no correlate with increased silver-resistance (Figure 10C).

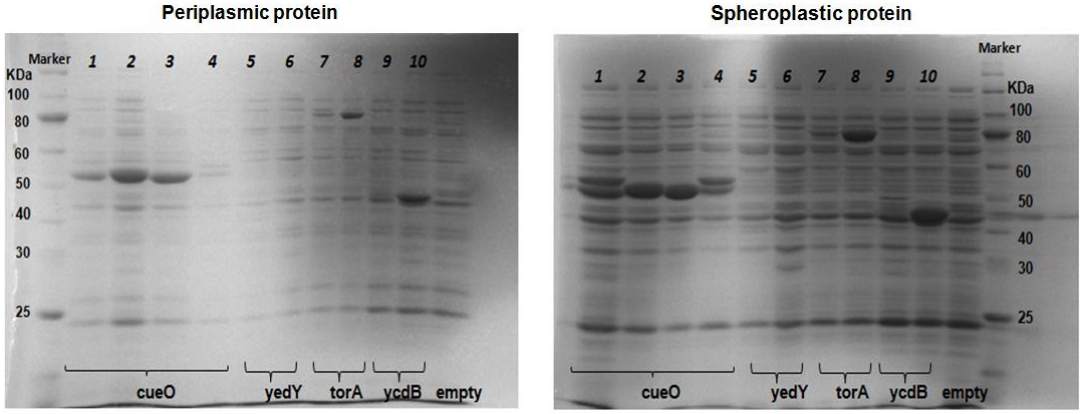

Figure 11. (A) SDS–PAGE analysis of Periplasmic and Spheroplastic proteins encoded by Tat genes. Pre (pre protein), Mat (mature protein), Molecular mass markers are indicated at the left. 1 CueO (58.404 KDa), 2 ΔT-CueO (55.474 KDa), 3 ΔTΔH-CueO (54.475 KDa), 4 ΔH-CueO (58.319 KDa), 5 YedY (39.318 KDa), 6 ΔT-YedY (34.569 KDa), 7 TorA (96.405 KDa), 8 ΔT-TorA (92.241 KDa), 9 YcdB (48.364 KDa), 10 ΔT-YcdB (45.061 KDa).

3.2.5 Comparing silver-resistance conferred by Tat genes under anaerobic and aerobic conditions

ycdB, *cueO* and *torA* were tested for resistance to metallic Ag(0) under aerobic and anaerobic conditions. Both in aerobic and anaerobic conditions, all three strains had increased resistance to metallic Ag(0) compared to the "empty control" (Figure 12). This cross-resistance to silver ion and

metallic Ag(0) supports the notion that both forms of silver exert their antimicrobial activity through common mechanisms.

In aerobic conditions, the antimicrobial activity of metallic Ag(0) was increased compared to anaerobic conditions, as expected from the oxygen-mediated dissolution of nanoparticles, albeit only slightly. Metallic Ag(0) appeared to be antimicrobial in all cases tested and killed bacteria in a time-dependent manner. In particular, no surviving colonies were found for the "empty" control under the conditions tested after only 4 hr incubation, even in an anaerobic environment where metallic Ag(0) would be expected to be largely stable. This observation suggests that there may be an antimicrobial mechanism of metallic Ag(0), independent of the air-mediated oxidation and concomitant release of silver ions. Alternatively, we cannot discount the possibility that these metallic Ag(0) preparations also contained some contaminating Ag^+, which could be the main antimicrobial agent in anaerobic conditions. *ycdB*, *cueO* and *torA* appear to be able to protect against both the silver ion-mediated killing and the postulated nanoparticle-selective mediated killing. *torA* expression conferred the most resistance to metallic silver, also in anaerobic conditions. It is noteworthy that the ratio of cfu in aerobic and anaerobic conditions differed between different strains (e.g. compare *ycdB* and *torA* in Figure 12)

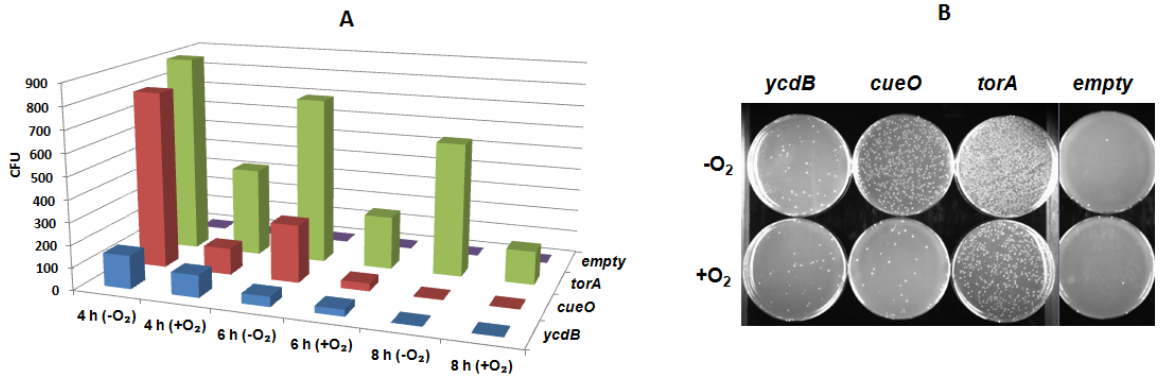

Figure 12. The resistance of strains expressing recombinant Tat-containing genes to 50 µg/ml metallic Ag(0) incubated at defined times under anaerobic and aerobic conditions. (A) Bar graphs of bacterial count surviving in the absence and presence of oxygen. (B) the corresponding Petri dishes after 6 h of incubation with 50 µg/ml metallic Ag(0).

3.3 Discussion

YcdB is a periplasmic haem-containing peroxidase-like protein and CueO is multicopper oxidase required for copper homeostasis: both these Tat-containing proteins were described as conferring resistance to silver in Chapter 2. By screening strains expressing each of the identified Tat-containing genes of *E. coli* for silver-ion resistance, we identified several genes -*sufI*, *hybA*, *yedY* and *torA*- which gave more resistance to silver ion than *cueO*. Of these, *torA* and *hybA* exhibited comparable resistance to silver ion to *cysD* (Figure 9), described at length in Chapter 2. The function of the 7 proteins encoded by Tat genes conferring most resistance to silver is listed simply in Table 5. Why do these Tat signal genes confer more resistance to silver?

SufI is structurally related to the multicopper oxidase superfamily, but analysis of the SufI sequence indicates that canonical copper-binding residues found in multicopper oxidases is almost absent: SufI is reported not to bind any copper and metal cofactors.[17] SufI is a water soluble and monomeric protein, which does not require insertion of a cofactor during folding. The overall structure of SufI is very similar to that of CueO and contains three cupredoxin-like domains. A further difference between the CueO and SufI structures is the absence, in SufI, of the methionine-rich helix and loop, the so-called tower of the CueO structure. So SufI is neither a copper-binding protein nor an enzyme. A role for SufI is postulated to be protecting and stabilizing the divisional assembly under conditions of stress and to serve as a scaffolding protein that helps to maintain the coherence of the septal ring during constriction in cell division: *i.e.* the protein SufI (FtsP) of *Escherichia coli* has been recognized to be a component of the cell division apparatus. It has been reported that silver ions, besides their effects on bacterial enzymes and DNA, also caused marked inhibition of bacterial growth and were deposited in the vacuole and cell wall as granules.[17] Silver inhibited cell division and damaged the cell envelope and contents of bacteria.[18] Cell division was disrupted at the initial stage and caused the cells to undergo morphological changes and die at the later stage.[19] However expression of SufI relieves the mytomycin C and paraquat sensitivity of division mutants, *i.e.* protects against radical-mediated damage,[20,21] so can remedy the radical-mediated damage caused by silver ion. How this anti-radical activity of SufI is achieved is unclear, but it is in broad agreement with conclusions of Chapter 2, which supports the oxygen radical-mediated killing of silver and a concomitant mechanism of silver-resistance by preventing the generation of oxygen radicals.

YedY was identified as the soluble periplasmic catalytic subunit of an oxidoreductase and contains a molybdo-molybdopterin cofactor. The overall fold of YedY is surprisingly similar to domain II of the eukaryotic chicken sulfite oxidase (CSO) and *Arabidopsis thaliana* sulfite oxidase (PSO). However, YedY shows a catalytic activity more in keeping with a reductase rather than a sulfite oxidase enzyme, because in YedY, only the IV and V states of molybdenum are attainable under the conditions reported.[22] YedY functions as a reductase for substrates including trimethylamine N-oxide (TMAO), Me_2SO, phenylmethyl sulfoxide, methionine sulfoxide, and tetramethylene sulfoxide. The apparent one-electron capacity of YedY may be able to catalyze the reduction of S- and N-oxides. It is tempting to postulate that the anti-silver ability of YedY may arise from the reduction of Ag(I) to Ag(0) by the Mo (V). However, this hypothesis would need experimental testing.

YcdO is also periplasmic, consisting of a C-terminal peptidase-M75 (M75) domain with a conserved 'HxxE' motif potentially involved in metal binding and an N-terminal domain that appears to resemble the copper-containing cupredoxins. The *E. coli* YcdO cup domain identifies two potential metal-binding sites. Site I is predicted to bind Cu^{2+} using three conserved residues (C41 and 103, and E66) and M101. Site II most probably binds Fe^{3+} and consists of four well conserved surface Glu residues. It is noteworthy that YcdO and YcdB appear to be organized in one operon with the gene *ycdN*. YcdB was also identified as conferring resistance to silver (see Chapter 2) and contains a noncovalent heme cofactor, which is assembled in the cytoplasm.[23] The anti-silver ability of *ycdO* is weaker than for other Tat genes and *cysD*. We speculate YcdO may be binding the monovalent silver ion, perhaps through its recognised copper (II) and iron (III) binding sites, without any involvement of catalytic activity, thus explaining its low resistance-conferring ability.

The HybA protein participates in the periplasmic electron-transferring activity of hydrogenase 2 during its catalytic turnover.[24] Because structural and functional information is largely unavailable for this protein, we shall abstain from speculating on possible mechanisms of resistance to silver conferred by this HybA protein.

The *E. coli* TMAO (trimethylamine N-oxide) reductase TorA is a soluble periplasmic enzyme containing the MGD (molybdopterin guanine dinucleotide) cofactor at its active site. TorA is a member of the DMSO reductase family of molybdoenzymes and the main respiratory enzyme responsible for TMAO reduction when the cells are grown anaerobically in the presence of TMAO;[25] therefore TorA plays an important role in energy conservation under anaerobic growth conditions. The TorA protein is encoded by the torCAD operon. TorA is located in the periplasm and receives electrons from TorC. The acquisition of molybdo-cofactor is a prerequisite for TorA translocation.[25] We speculate that the anti-silver ability of TorA may be similar to YedY, which also contains the molybdo-molybdopterin form of the molybdenum cofactor. Both these proteins are reductases that can reduce TMAO. CueO's function is strictly oxygen-dependent, even though expression of the *cueO* gene also occurred under anaerobic conditions.[26]

All genes discussed in this chapter are Tat substrate proteins. At the beginning of this chapter we proposed the assumption that Tat signal sequence might play an important role in the resistance of proteins to silver ion. However, our results suggest that the Tat signal sequence only has small effect or no effect on anti-silver ability of these proteins. Silver resistance appears to be conferred by the activity of the proteins themselves (Figure 10), because expression of Tat or His-Tat alone did not lead to any significant resistance to silver compared to controls. However, Tat was not totally essential for export: especially for CueO and TorA, deletion of Tat did not affect export nor protection from silver. In all these proteins, deletion of Tat did not totally eliminate resistance, either because the recombinant proteins exert also a protective mechanism in the cytoplasm, but more likely because they are still partially exported (perhaps co-exported) even without Tat, a phenomenon that is well documented in the literature.[27]

Table 5. The basic functions of the 7 Tat genes selected.

Name	Function	Reference
hybA	Participates in the periplasmic electron-transferring activity of hydrogenase 2 during its catalytic turnover.	28, 29
torA	Reduces trimethylamine-N-oxide (TMAO) into Trimethylamine; an anaerobic reaction coupled to energy-yielding reactions.	29, 30
yedY	The exact function is not known. Can catalyze the reduction of a variety of substrates like dimethyl su lfoxide, trimethylamine N-oxide, phenylmethyl sulfoxide and L-methionine sulfoxide. Cannot reduce cyclic N-oxides. Shows no activity as sulfite oxidase.	29, 31
sufI	Cell division protein that is required for growth during stress conditions. May be involved in protecting or stabilizing the divisomal assembly under conditions of stress.	20, 21
ycdO	Involved in Fe^{2+} uptake. Could be an iron-binding and/or electron-transfer component.	29, 32
cueO	Probably involved in periplasmic detoxification of copper by oxidizing Cu^{+} to Cu^{2+} and thus preventing its uptake into the cytoplasm. Possesses phenoloxidase and ferroxidase activities and might be involved in the production of polyphenolic compounds and the prevention of oxidative damage in the periplasm.	25,33
ycdB	Involved in the recovery of exogenous heme iron. Extracts iron from heme while preserving the tetrapyrrol ring intact. Also displays peroxidase activity on guaiacol in vitro.	29, 34

3.4 Conclusion

Our results demonstrated that expression of some Tat genes also endow more resistance to silver. Taken together, we conclude from the exploration of these recombinant Tat proteins by probing constructs with defined deletions for resistance to silver: 1) deleting the Tat signal sequence of genes has little effect on silver resistance, typically leading to marginally more susceptibility of the strains; however, these constructs typically expressed more recombinant protein, which was often still exported to the periplasm; *i.e.* the Tat signal sequence was not totally essential for export; 2) deleting the His tag of genes had little effect or actually increased resistance, thereby excluding the possibility that export of silver ions bound to the His-tag via the Tat pathway is a main mechanism of resistance; the His-tag sometimes affected expression and export of Tat proteins (e.g. for YedY and CueO); 3) deleting insert genes -expressing only Tat sequences with or without a His tag- drastically decreased the anti-silver ability of the host., *i.e.* the anti-silver ability of Tat genes was mainly from the insert genes but not from the His-tag or Tat-sequences, either alone or in combination. YcdB, CueO and TorA appear to be able to protect against both the silver ion-mediated killing and the postulated nanoparticle-selective mediated killing (which has, to our knowledge, never been demonstrated conclusively). The exact mechanism by which these enzymes exert their protective action against silver remains to be elucidated, although we believe that it may be mediated by their redox activities in the periplasm. It may be interesting to explore the resistance of bacteria expressing defined catalytic mutants of these enzymes.

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Chapter 4 Directed evolution of genes for increased resistance to silver

Introduction

Previous chapters have described the identification of genes of *E. coli* whose recombinant overexpression leads to increased persistence in the presence of silver ion or nanoparticles. The goal of this chapter was to determine whether the silver-resistance function of some of these genes could be improved using directed evolution, as a means to gain insight into how resistance may function and evolve.

Directed evolution is a method used in protein engineering, by which the process of natural selection to evolve proteins or nucleic acids are mimicked, typically on single genes, on a laboratory timescale, and by which special properties can be explored, which are not necessarily found in nature.[1-4] For example, investigators have used directed evolution to improve or alter thermal and chemical stability where these specific traits are not apparent in the parent molecule,[5] for novel protein-based tools and devices or to enhance the promiscuous activities of enzymes toward altered specificity.[6-10] The directed evolution process is based on the generation of mutant gene libraries, from which further improved mutants can be isolated by screening or selection assays. In directed evolution experiments creating molecular diversity is a crucial step. Routinely, diversity of proteins is now achieved by targeted or by random mutagenesis of the gene encoding an existing protein, either through altering the reaction conditions of standard PCR or through the use of error-prone DNA polymerases. The changed gene is introduced into a bacterial cell by transformation of a plasmid. This mutant gene produces the particular protein mutant.

In this chapter, we chose 4 genes, which had been previously selected for resistance of AgNPs and silver ion in the absence and presence of IPTG, to conduct the directed evolution experiments in order to improve the anti-silver ability of these genes. The 4 genes chosen were *ycdB*, *ydgT*, and *iscA* (all originally selected without IPTG induction) and *yeaO* (selected with IPTG). Genes *ydgT*, *yeaO* and *iscA* conferred low resistance to silver, but barely above background, whereas *ycdB* conferred a little more resistance and was used to explore whether it is easy to improve further its anti-silver ability. In the experiments described in this chapter, random mutations were introduced into copies of *ycdB*, *ydgT*, *yeaO* and *iscA* genes by error-prone PCR. The mutagenized inserts were ligated into the expression vector modified pCA24N, which carries a chloramphenical resistance marker. Mutants with improved anti-silver ability were selected at the concentration of silver ion that identify mutants with enhanced resistance at LB medium supplemented with IPTG (Figure 1).

The YdgT protein is an unkown protein and thought to be paralogue of Hha in *E. coli*.[11] The Hha and YdgT proteins have been found to adjust the expression of horizontally acquired genes by interacting with H-NS and StpA, which play primary roles in the transcriptional silencing of such genes.[11] Overexpression of YdgT in hha mutants compensates at least partially some hha-induced phenotypes, in a similar way as it was described for the paralogue proteins H-NS and StpA. The interaction between Hha and H-NS families of proteins demonstrate that both Hha and YdgT are capable of generating heteromeric complexes with H-NS and StpA. Interaction of StpA with either Hha or

YdgT can also protect the former from Lon-mediated proteolysis.[12] In our reasearch YdgT was chosen as the candidate of directed evolution only because it was present in many different clones during selection without IPTG (Chapter 2), although validation failed to show unequivocally whether it conferred increased resistance to silver.

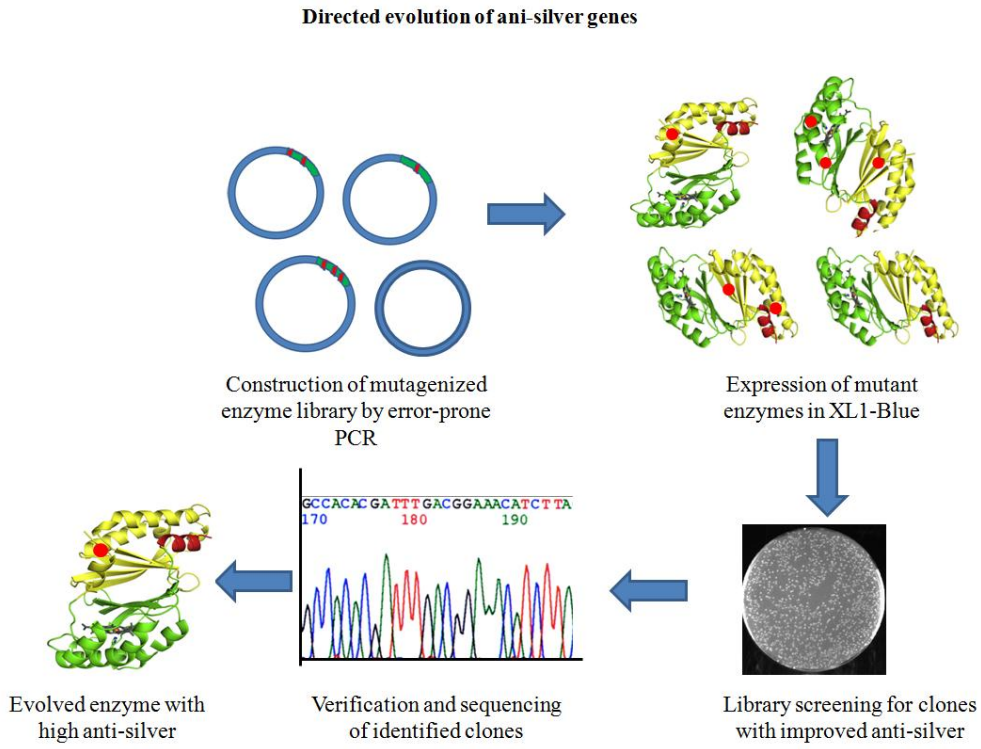

Figure 1. General strategy for directed evolution of genes conferring improved resistance to silver.

IscA is a key member of the iron-sulfur cluster assembly machinery found in bacteria and eukaryotes. It can bind iron and [2Fe-2S] clusters, and participates in the biosynthesis of iron-sulfur proteins. IscA belongs to 110 amino acid residues long of scaffold proteins, which contain three conserved Cys residues in the sequence motif Cys-Xn-Cys-X-Cys (n=60–80), which are critical for Fe-S cluster binding and therefore its function. The Fe-S complexes of IscA are labile, and experiments *in vitro* have shown that clusters can be transferred from IscA (Fe-S) to apo-protein forms of other iron-sulfur proteins.[13,14] The function of IscA, however, is not well understood. IscA may act as an iron chaperone that delivers iron for the biogenesis of iron-sulfur clusters in IscU. Because IscA had been found in our preliminary selection experiments but conferred only very low resistance ability to silver, it was chosen for directed evolution. Finally, YeaO is a hypothetical protein with unknown structure and function. YeaO was chosen for directed evolution because it was selected with AgNPs and silver ion in the presence of IPTG, although it conferred very little, but detectable resistance to silver.

4.1 Material and methods

All chemicals were purchased from Applichem, unless otherwise specified. The polymerase and other restriction enzymes are ordered from New England Biolabs, unless specified. Modified

pCA24N, 4478bp, was used as the vector backbone, which includes two restriction sites *NcoI (C/CATG/G*) and *NotI* (*GC/GGCC/GC*). Forward primer Nco1 restriction site (5' A TTA ACC ATG GGA GGA TCT CAC CAT CAC C 3'), reverse primer (5' GGA GTT CTG AGG TCA TTA CTG G 3') were synthesized from Microsynth, Switzerland and the gene sequencing was from Starseq, Germany.

4.1.1 Creating a mutant library

A single round of error-prone PCR was performed using the GeneMorph Ⅱ Random Mutagenesis Kit (Agilent technologies), which employs Mutazyme Ⅱ DNA polymerase (a blend of Mutazyme I DNA polymerase and a novel Taq DNA polymerase mutant) that exhibits increased misinsertion and misextension frequencies compared to wildtype Taq. The combination of these two polymerases is thought to reduce bias and produce equivalent mutation rates at AT and GC sites, therefore promoting variation in the mutation type.

The error-prone PCR reaction mixture (50 µl final volume) is comprised of: 41.5 µl of water, 5 µl of 10× Mutazyme II reaction buffer, 1 µl of 40 mM dNTP mix (200 µM each final), 0.5 µl of primer mix (250 ng/µl of each primer), 1 µl of Mutazyme II DNA polymerase (2.5 U/µl), 1 µl template containing the DNA that was to be mutagenized. Forward primer *Nco*1 restriction site (5' A TTA ACC ATG GGA GGA TCT CAC CAT CAC C 3'), reverse primer (5' GGA GTT CTG AGG TCA TTA CTG G 3') were used. The amount of target DNA recommended by manufacturer here is 0.5-1µg in order to gain low mutation rate (0-4.5 mutations/kb), so the amount of each plasmid template added should be calculated according to initial target DNA amount required for the mutation frequency desired. In the PCR reaction, all target DNA amount added were 0.5 µg. pCA24N vector[15] (4478 bp). The target DNA *ycdB* was 392559 g/M, therfore the amount of plasmid *ycdB* added was 0.5×(392559+1619925)/392559 µg. For *ydg*T, *iscA* and *yea*O, the plasmids added were 0.5×(64671+1619925)/64671 µg, 0.5×(98808+1619925)/98808 µg and 0.5×(105652+1619925) /105652 µg respectively. The error-prone PCR was run following the conditions: hot start PCR, with initial 95°C for 2 minutes, then 30 cycles of 95°C/1 minute, 56°C/1 minute and 72°C/2 minutes, with a final extension of 10 minutes at 72°C. The error-prone PCR product (2 µl) was run on an ethidium bromide-stained agarose gel alongside a DNA ladder. The remaining PCR product was digested to eliminate the template plasmids with *Dpn*I (5 µl) for 5 h, and then the products was purified by PCR clean-up kit. The purified products were further digested with *Nco*I and *Not*I enzyme (µl) in NEBuffer 3.1 to prepare the insert genes.

The modified pCA24N vector backbone (Derivative of pCA24N, where one T->C and A->G mutation places a *NcoI (C/CATG/G)* cl**o**ning site, producing cohesive ends. with *Nco*1/*Not*1 insertion from ASKA library) was used for the cloning and expression of error-prone library. In order to obtain the vector backbone, pCA24N was digested with *NcoI* and *NotI* enzyme in NEBuffer 3.1 in 1 hour at 37°C. The insert genes and vector pCA24N digested were purified by PCR clean-up kit to ensure the ligation success. After that the insert mutagenesis library genes and vector pCA24N were ligated at a 5:1 molar with T4 DNA for 2.5 h at 37°C. After obtaining mutagenesis plasmid library we transformed this whole library into XL1-Blue with electroporation in order to get the libraries of mutant colonies for selection.

4.1.2 Selection of mutants with high anti-silver ability

After obtaining the mutant bacterial library, we collected all colonies with LB medium (more than 1000 colonies) and used pooled colonies as preculture. Further, the mutant colonies were selected according to methods described in previous chapters. Briefly, the cultures containing preculture, chloramphenicol and tetracycline (5 and 34 µg/ml) in the presence of IPTG were incubated to OD_{600} 0.4 at 37°C. Adding 7 µg/ml silver ion supplied as silver nitrate (to select the mutant genes with high anti-silver ability we increased the concentration of silver ion from 6 to 7 µg/ml) to OD_{600} 0.4 ASKA strains and incubated the culture 7 h at 25°C. After 7 h of incubation with silver ion. 20 µl of culture was diluted 100 times, from which 100 µl spread on every plate. All the plates were incubated for 24 h at 37 °C. Plasmids were purified from some of the resulting colonies, for re-transformation and re-validation of the resistance to silver ion using 7 µg/ml of silver ion supplied as silver nitrate. The experimental process was the same as with the selection process, *i.e.* adding 7 µg/ml silver ion to OD_{600} 0.4 ASKA strains and incubated the culture 7 h at 25°C. After 7 h of incubation with silver ion. Ten (10) µl of culture was diluted 100 times and 100 µl of this dilution was spread on every plate. All the plates were incubated for 24 h at 37°C. All picked colonies from the selected colonies were sequenced to check for mutations in the gene insert. In order to analyse the mutagenesis protocol, 10 colonies were also picked at random from media plates in absence of silver-selection and their gene inserts sequenced from the purified plasmids.

After obtaining the mutants with high resistance to silver ion, we also examined resistance ability of these mutants to AgNPs and antibiotics. When 0.4 OD_{600} culture was obtained defined concentration of AgNPs and antibiotics were added into the culture and incubated for certain time at 25°C. Ten (10) µl of culture was diluted 100 times and 100 µl of this dilution was spread on every plate.

4.2 Results

In our directed evolution experiment, we wanted to improve the anti-silver ability of some genes by random mutagenesis of the gene. Mutagenesis and further selection of genes identified as conferring resistance to silver was designed to identify evolved mutants conferring further resistance to silver ion.

Table 1 Mutations in the *yeaO* variants selected in the presence of 7 µg/ml silver ion.

Mutant	Amino acid change	Phenotypes
1	Q118STOP	++
2	L131C	--
3	T61M and L131F	--
4	L131F	+
5	K103R and Q118STOP	-
6	E82V	--
7	C21F	+
8	K96T	+
9	F80V	-

'++' means more resistance than wildtype, '+' the same resistance as wildtype, '-' less resistance than wildtype, '--' much less resistance than wildtype. 14 colonies were sequenced, in which 9/14 were mutants and 5/14 were wildtype.

For the *yeaO* mutant library, sampling of the library selected with 7µg/ml led to the identification of 9 mutants (Table 1), all of which had one or two non-synonimous point mutations. Mutant 1 contained one premature nonsense mutation, which terminated the protein after 118 amino acids (13% of protein truncated). To confirm if these mutants of *yeaO* led to improved resistance to silver ion, a retransformation test was performed. We retransformed these mutant genes into XL1-Blue cell, then confirmed the improved phenotype of mutant strains by comparing them to that of T-*yea*O (template-*yeaO*), W-*yeaO* (wildtype *yeaO*, *i.e.* the original ASKA plasmid) and M-pCA24N (modified empty pCA24N, the cloning vector used for this experiment) (Figure 2). This validation showed that, whereas some of the mutants performed worse than the wildtype, mutant 1 (Q118Stop-*yeaO*) conferred increased resistance compared to the original *yeaO*. Another truncated gene, containing mutations K103R & Q118Stop, did not render the host more resistant, and it appears that the conservative mutation K to R at residue 103 was deleterious for function (Figures 2 and 3).

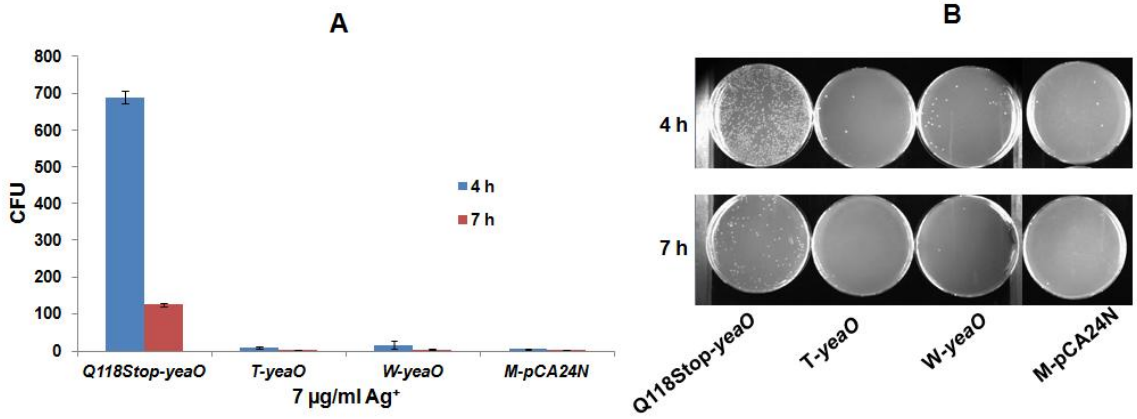

Figure 2. The resistance ability of *yeaO* strains to 7 µg/ml Ag^{+}. Q118Stop-*yeaO* presents glutamine of *yeaO* was changed to stop codon by the er-PCR. T-*yeaO* represent template *yeaO* used in the er-PCR reaction. W-*yeaO* is the wildtype *yeaO* from ASKA library. M-pCA24N is modified pCA24N. (left) bar graph and (right) Petri dishes of *yeaO* and vector strains after 4 and 7 h of incubation with 7 µg/ml Ag^{+}. Error bars represent means the SD (standard deviation) from three biological replicates.

```
T-YeaO              MGGSHHHHHHTDPALRANIQCKRVYDPAEQSDGYRILVDRLWPRGIKKTDLALDEWDKEI 60
Q118STOP-YeaO       MGGSHHHHHHTDPALRANIQCKRVYDPAEQSDGYRILVDRLWPRGIKKTDLALDEWDKEI
                    ************************************************************

T-YeaO              TPSTELRKAFHGEVVDYATFREQYLAELAQHEQEGKRLADIAKQPLTLLYSAKNTTQNH 120
Q118STOP-YeaO       TPSTELRKAFHGEVVDYATFREQYLAELAQHEQEGKRLADIAKQPLTLLYSAKNTT-NH
                    ********************************************************** **

T-YeaO              ALVLADWLRSLGLCGR 136
Q118STOP-YeaO       ALVLADWLRSLGLCGR
                    ****************
```

Figure 3. Sequence comparision of the mature domains of T-YeaO (Template-YeaO) and mutant Q118Stop-YeaO. The position of mutation is shown by red color and green color showed the position of mutation for lysine residue (to arginine) which occurred in mutant 5. The sequence underlined is artifical recombinant sequences introduced by the cloning strategy.

Using the same approach as described above for *yeaO* random mutations were introduced into the template *ydgT* gene by error-prone PCR. After this mutant library was transformed into XL1-Blue

we picked some colonies from the mutants strain library for sequencing and 8 mutants were obtained which were shown in Table 2. For all mutants only one amino acid was substituted. A retransformation test was performed again to confirm the resistance of these mutants of *ydgT* to silver ion. Only mutant 4 of *ydgT* improved the anti-silver ability of original gene of *ydgT* while the other mutants decreased the resistance of *ydgT* to silver ion (Figure 4). The number of surviving clones upon selection in presence of silver ion of Q84H-*ydgT* was almost 5 times higher than for other control strains. This confirms the change of Q to H amino acid (Figure 5) improved the resistance of the *ydgT* to silver upon induction with IPTG.

Table 2. Mutations in the YdgT variants selected in the presence of 7 µg/ml silver ion

Mutant	Amino acid change	Phenotypes
1	Y39N	--
2	D60A	--
3	V67A	--
4	Q84H	++
5	V67D	+
6	I53T	+
7	D21N	--
8	H41L	-

'++' means more resistance than wildtype, '+' the same resistance as wildtype, '-' less resistance than wildtype, '--' much less resistance than wildtype. 25 colonies were sequenced, in which 8/25 are mutants and 17/25 are wildtype.

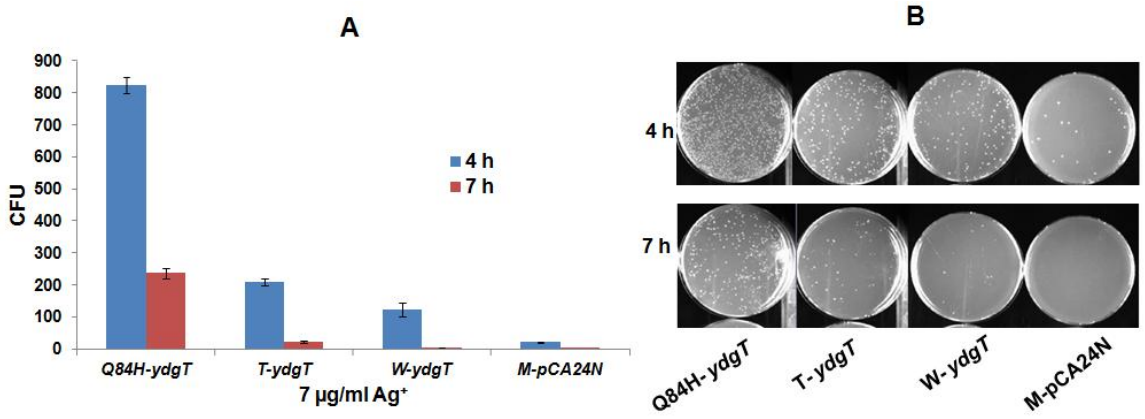

Figure 4. The resistance ability of *ydg*T strains to 7 µg/ml Ag^+. Q84H-*ydgT* presents glutamine of *ydgT* was changed to histidine by the er-PCR. T-*ydgT* represent template YdgT used in the er-PCR reaction. W-*ydgT* is the wiltype *ydgT* from ASKA library. (left) bar graph and (right) Petri dishes of *ydgT* and vector strains after 4 and 7 h of incubation with 7 µg/ml Ag^+. Error bars represent means SD (standard deviation) from three biological replicates.

For *iscA*, we picked some colonies from the *iscA* variant library to sequence. Twelve (12) mutants were identified (Table 3), all bearing a single nucleotide change. However, validation experiments by retransformation and reselection at 6 or 7 µg/ml Ag^+ failed to detect variants with increased resistance compared to wildtype: most of the mutants had less resistance than wildtype and only one had similar resistance.

A

```
T-YdgT          MGGSHHHHHHTDPALRATVQDYLLKFRKISSLESLEKLYDHLNYTLTDDQELINMYRAA 60
Q84H-YdgT       MGGSHHHHHHTDPALRATVQDYLLKFRKISSLESLEKLYDHLNYTLTDDQELINMYRAA
                ************************************************************

T-YdgT          DHRRAELVSGGRLFDLGQVPKSVWHYVQGLCGR 93
Q84H-YdgT       DHRRAELVSGGRLFDLGQVPKSVWHYVHGLCGR
                ************************:***:*****
```

B

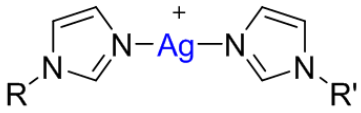

Figure 5. (A) Sequence comparison of the mature domains of T-YdgT (Template-YdgT) and mutant Q84H-YdgT. The position of mutation is shown by red color and green color showed another histidine residue close to the position of mutation. (B) Chemical structure of a Ag(I)-mediated "histidine-bridge" (ref 17). The sequence underlined is artifical recombinant sequence introduced by the cloning strategy.

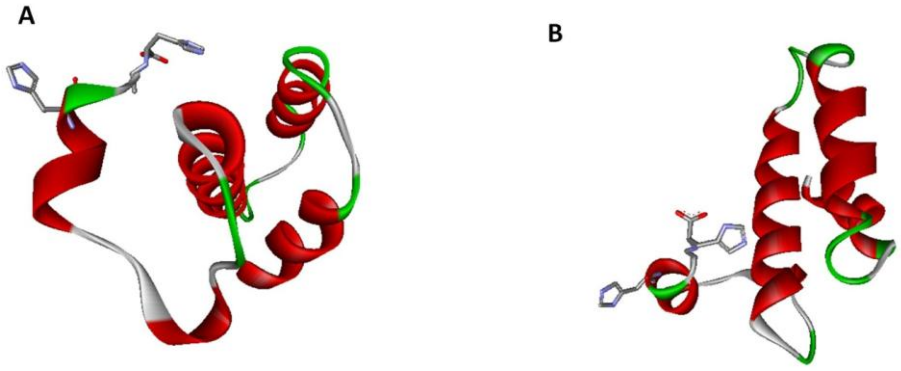

Figure 6. Homology model of mutant Q84H-YdgT. The two views (A) and (B) are rotated 90° from each other. The two vicinal histidines, including the one at position 84 of the mutant right at the C-terminus of the model, are shown. This C-terminal region is thought to be flexible and is not thought to interfere with binding to the protein partner H-NS.[1] The model is generated using the automated Swiss-Model software using the structure of the ortholog protein hha as template (pdb:4ICG).

Overall, in the 14 mutants sequenced 9 wildtypes were found. Although more mutants were acquired for *iscA* than *ydgT* and *yeaO* no single mutant was found that improved the anti-silver ability of *iscA*. These findings suggest that the function and activity of IscA protein may difficult to be enhanced only by one single round of error-prone PCR. Finally, we identified only two mutants of *ycdB*, but these also failed to confer enhanced anti-silver ability in validation experiments. No further mutants were identified upon sequencing several dozen unselected plasmids from this *ycdB* library, suggesting that the mutation efficiency was low for *ycdB*.

Table 3. Mutations in the IscA variants selected in the presence of 7 µg/ml silver ion

Mutant	Amino acid change	Phenotypes
1	G3E	-
2	M54T	--
3	M54K	+
4	Y56C	--
5	P66L	-
6	G45D	-
7	S49F	-
8	I19T	-
9	V76M	-
10	L33Q	-
11	I19T	-
12	F106L	-

'++' means more resistance than wildtype, '+' the same resistance as wildtype, '-' less resistance than wildtype, '--' much less resistance than wildtype. 21 colonies were sequenced, in which 12/21 are mutants and 9/21 are wildtype, others were disordered.

Table 5. Mutations in the *ycdB* variants selected in the presence of 7 µg/ml silver ion

Mutant	Amino acid change	Phenotypes
1	S4Y and Q275A	-
2	Y19C	-

'++' means more resistance than wildtype, '+' the same resistance as wildtype, '-' less resistance than wildtype, '--' much less resistance than wildtype. 4 colonies were sequenced, in which 2/4 are mutants and 2/4 are wildtype.

Mutants Q118Stop-*yeaO* and Q84H-*ydgT* have improved resistance ability to silver ion, so we further tested the resistance of these two mutants to AgNPs and antibiotics (Figure 7). By comparison it can be seen these two mutants Q118Stop-*yeaO* and Q84H-*ydgT* also have improved resistance to 200 µg/ml of AgNPs (Figure 6A and B). However the results of resistance of these two to antibiotics (ampicillin and kanamycin) did not show improved resistance compared to control (Figure 6C). This indicated that mutants evolved by silver ion can also give resistance to different silver compounds –*i.e.* to silver nanoparticles- but that increased resistance to silver does not necessarily lead to increased resistance to antibiotics.

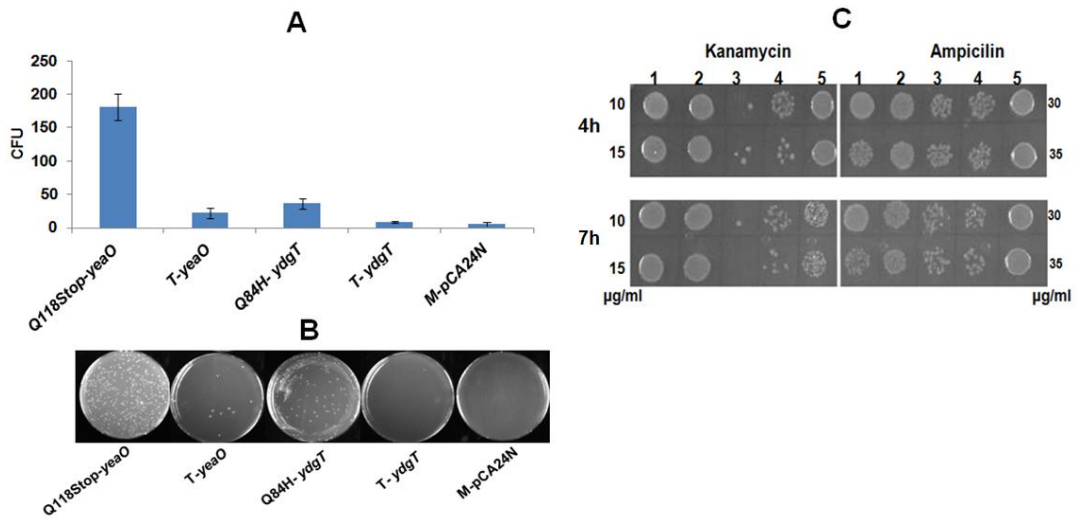

Figure 7. The resistance ability of *yeaO* and *ydg*T strains to AgNPs and antibiotics. (A) bar graphs of resistance of two mutants to 200 μg/ml of AgNPs after 3.5 h of incubation. (B) corresponding Petri dishes of resistance of two mutants. (C) The resistance ability of *yeaO* and *ydg*T strains to 10 and 15 μg/ml of kanamycin and 30 and 35 μg/ml of ampicilin. 1 w-*yeaO*, 2 Q118Stop-*yeaO*, 3 w-*ydgT*, 4 Q84H-*ydgT*, 5 M-pCN24N. Error bars represent means SD (standard deviation) from three biological replicates.

4.3 Discussion

In the 1970's Ohno hypothesized that duplication underlines molecular evolution of new functions,[16] since after duplication one gene would be entirely redundant and freed from all constraints, to either become a nonfunctional pseudogene in most cases, with little loss of fitness for the organism, or to evolve a new function with a concomitant gain of fitness. In this chapter, we aimed to improve the anti-silver resistance function of *yeaO*, *ydgT*, *iscA* and *ycdB*.

In our directed evolution experiment, on the basis of Ohno's hypothesis, first we selected for silver resistance conferred by copies of genes of *E. coli* borne by a plasmid –with the original gene maintained in the chromosome of the host. Further mutation and selection of the plasmid-borne genes was then made possible, thus evolving for a new activity without loss of original function of the chromosomically-encoded gene. Although this directed evolution was laboratory-based, we suggest that it may have parallels with what may be happening in the "wild", for example during selection pressure imposed by use of antimicrobial silver in clinical environments.

We found that the anti-silver resistance of *yeaO* and *ydgT* could be improved by single amino-acid replacement mutation or truncation of the genes. For *yeaO*, only the nonsense mutant Q118STOP-*yeaO* was found to improve resistance to silver, whereas another truncated mutant with K103R mutation failed to show improved resistance. The reason why the nonsense mutant Q118STOP-*yeaO* can improve the anti-silver ability is still very difficult to predict without further structural and biochemical information. The protein *yeaO* is currently labeled as "hypothetical protein" and the structure and function are not known.

For the mutants of *ydgT*, the Q84H (Figure 5) substitution conferred enhanced silver-resistance. Histidine is an amino acid containing an imidazole side-chain and it is widely recognized that Ag^+ can bind to imidazoles[17,18] and that the moderate Ag(I)-N bonding property may play an important role in showing the wide spectra of antimicrobial activities of silver.[19] Silver(I) atom is known as a soft Lewis acid and has a relatively low affinity for hard oxygen donors, high affinities for soft donors S and P, and moderate affinities for nitrogen donors, and it usually forms two- or four-coordination complexes.[20] For the bonding ability of silver(I) atom, some experiments in ligand-exchange reactions have suggested the relative order of strength to be: Ag(I)-S >> Ag(I)-C1 > Ag(I)-N >> Ag(I)-O.[21] It is of interest that the newly-introduced histidine in the mutant Q84H is close to another histidine residue, in what may be a flexible region at the C-terminus of this small protein (Figure 6). Taken together, we speculate that the two histidine residues of mutant Q84H-YdgT may coordinate silver ions by the two imidazole motif. However, since histidine-tag overexpression appears insufficient to confer increased resistance to silver, the Q84H mutation of YdgT may also be increasing the biological function of this protein.

In contrast to YdgT and YeaO, we did not find any mutants of YcdB and IscA conferring increased resistance to silver. For *ycdB*, only got very few transformants were obtained and larger libraries should be tested. We conclude from the experiments with *iscA*, where comparable libraries to *ydgT* and *yeaO* were obtained, that it may be challenging to select for improved variants. The protein encoded by *iscA* is a key member of the iron-sulfur cluster assembly machinery and participates in the biosynthesis of iron-sulfur cluster proteins. Most mutations that we explored led to increased susceptibility to silver, indicating that the anti-silver ability of *iscA* is difficult to improve. Measurement of mutants Q118Stop-*yeaO* and Q84H-*ydgT* to AgNPs and antibiotics verified that these two mutants have cross resistance to silver ion and to AgNPs, but not to antibiotics. This result is encouraging because, despite the finding that resistance to silver can correlate to resistance to antibiotics (see chapter 2), it suggests that this dependency is not absolute and that evolution of silver-resistance does not necessarily lead to increased resistance to antibiotics.

4.4 Conclusions

Evolution is a very powerful and useful design strategy. In this chapter we improved genetically the silver resistance conferred by two plasmid-encoded genes by laboratory evolution. We reach two main conclusions: first, our facile proof-of-concept evolution of two genes for silver ion or nanoparticle resistance suggests that silver-resistance may also evolve easily outside the laboratory, such as in hospitals or other environments; indeed, we suggest that low level resistance by overexpression of defined genes initially conferring low-level resistance (*e.g.* due to duplication or plasmid-encoded multicopy) offers a first step toward the evolution of increased resistance, whether in the laboratory or in the wild. Second, evolution of silver-resistance in these two gene-variants did not correlate to increased antibiotic resistance. This latter finding offers hope that widespread use of antimicrobial silver will not hinder necessarily the urgent fight against antibiotic resistance. On the contrary, the evolved variants discovered could even prove of practical interest, for example in their potential future applications in recombinant bacterial strains for the purposes of bioremediation. Further to the proof-of-concept of evolution of resistance to silver described here, more work will be needed to explore the structure and function of mutants and to elucidate the mechanism by which they confer their resistance to silver.

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Chapter 5 Summary and Outlook

With the rising incidence of antibiotic resistance and the emergence of new virulent pathogens, there is a growing need to enhance our antibacterial arsenal. This is particularly imperative for infections caused by Gram-negative bacteria.[1-3] Silver is known as a board spectrum antibacterial agent. Despite this long-standing history and its demonstrated activity against Gram-negative bacteria, the complete bactericidal mode of action of silver remains unclear. In order to research these issues, we first identified anti-silver genes, including *cysD* and *ycdB*, by selection in presence of silver ion or nanoparticles of *E.coli* expressing the ASKA plasmid library, which comprises each open reading frame of the genome of this bacterium. The *cysD* gene not only gave more resistance to kinds of silver compounds but also rendered cells more resistant to Cu^{+} and Cd^{2+}. Silver ions and nanoparticles were more toxic in aerobic than anaerobic conditions, suggesting that nanoparticles can release toxic silver ions, a view that is reaching an increasing consensus in the field. Assays for production of hydroxyl radicals also support the notion that toxicity is mediated through reactive oxygen radicals (ROS) and that prevention of ROS production is involved in the acquisition of resistance. We also tested the resistance of *cysD* and *ycdB* expression to the antibiotics kanamycin and ampicillin as well as synergistic function of antibiotics and silver. Our results suggest that anti-silver genes can also give rise to resistance to antibiotics. However, we also found that kanamycin and silver act synergistically and that the combination of both compounds tremendously improved the antibacterial activity of both antimicrobial agents; in contrast, such synergism was not observed between ampicillin and silver. Our search for genes conferring silver resistance uncovered a surprising array of proteins, some of uncharacterised. The finding that overexpression of several Tat-exported proteins, most particularly TorA, led to considerable silver-resistance points toward redox processes within the periplasmic space of Gram-negative organisms being crucial to the antimicrobial activity of silver. Finally, we provide proof-of-concept using directed evolution of two different genes (*ydgT* and *yeaO*) that increased silver resistance is evolvable from low-level resistance by incorporation of point mutations. This finding illustrates that anti-silver mutants could potentially be obtained also in hospital or any other environments where there is selection pressure due to silver toxicity. However, in the case of *ydgT* and *yeaO,* increased resistance to silver did not correlate with increased resistance to kanamycin nor ampicillin. This latter preliminary observation offers the hope that increasing use of silver in consumer and medical products will not necessarily contribute to antibiotic resistance.

The work presented here, however, leaves many open questions, including:

- Do nanoparticles contribute to toxicity in the absence of silver ion production? Although all evidence points toward ionic silver being the main toxic species, it remains unclear whether silver nanoparticles themselves contribute at all to toxicity. We were unable to find any gene whose overexpression led to resistance to any one particular form of silver alone, *e.g.* resistance to nanoparticles alone or resistance to silver ion alone. The finding of generalized cross-resistance further supports that silver ion and silver nanoparticles share a common mechanism of toxicity.
- Stoichiometric or catalytic? We speculate that the low-level resistance of the *ydgT* mutant harbouring an additional histidine, may be due to direct binding to silver, a hypothesis that requires further structural and biochemical exploration. However, the finding that several

proteins expressed at low level contribute to considerable silver resistance, such as *cysD* and *ycdB*, points toward the existence of catalytic mechanisms of resistance, rather than simply *via* direct silver-coordination. Supporting this view is the observation that expression of his-tag, whether alone or fused to a tat-signal peptide, was insufficient to confer significant resistance to silver. It may be interesting to explore the resistance of bacteria expressing defined catalytic mutants of these enzymes.

- How does *cysD* confer resistance to silver? In our hands, expression of *cysD* led to most silver resistance, although it was expressed at low levels. Nevertheless, we found that extracts of the cysD expressing strain did not have increased levels of thiols, as could be expected from its involvement in cysteine metabolism. However, quantification of hydroxyl radical production suggested that both the *cysD* and *ycdB* expressing strains had lower levels of reactive oxygen species (ROS) than equivalent controls, although they showed no increased resistance to hydrogen peroxide. Together, these observations suggest that prevention of ROS generation, possibly mediated through protective sulfur-containing compounds, may lead to increased resistance to silver. Here, metabolomic profiling of the various strains could prove informative.
- What is the role of redox processes in silver toxicity? Exposure to silver led to increased ROS production, which correlated with increased bacterial killing. However, exactly how ROS were generated remains unclear. At first glance, oxidation of Ag(0) to Ag^{+} requires a loss of an electron, which may lead to oxygen radical generation. From this point of view, it may not necessarily be the nanoparticle or the ion *per se* that is the toxic species, but rather the radical generated. However, further investigation makes it clear that it is the most soluble silver salts that are the most toxic of any silver species tested and, moreover, we found that silver ion itself led to the generation of ROS.[4] However, how does silver ion lead to increased generation of ROS? Is silver itself participating in redox processes, or is it replacing other bound metal species, such as copper or iron, which can then participate in Fenton-like processes? The finding of cross-resistance of *cysD* and *ycdB* expressing strains to Cd^{2+} suggests the latter.
- What is the role of redox processes in silver resistance? As noted above, expression of *cysD* and *ycdB* led to lower levels of ROS and increased resistance. Several of the genes selected, particularly the Tat-signal encoding genes, are reported to encode enzymes that catalyze redox chemistries. Therefore, there is an emerging picture that prevention of radical formation may lead to increased resistance to silver. However, how is this putative prevention of ROS formation achieved, exactly? Is it through quenching radical species? Is it through stabilization of toxic metals to redox-inert species or through formation of metallic silver through reduction of silver ion? Could some of the strains of *E. coli* described here be *forming* silver nanoparticles? What is the main oxidized species in the postulated silver ion-mediated Fenton chemistry and what is the final "electron sink" in susceptible and in resistant hosts?
- What is the physiological function of the genes identified to confer silver resistance? For several of the genes found, the structure and function remain unknown. For example, virtually no information on structure and function is available for *yeaO*. However, expression of a mutant of this hypothetical protein with a premature stop codon (leading to a

truncated protein 18% shorter) conferred markedly increased resistance to silver. Clearly, further structural and biochemical data are required.

- Why does the silver and kanamycin combination lead to such synergy in antimicrobial action (but not silver and ampicillin)? Could this finding be clinically useful? Are there other such synergisms with potential to lead to better antibiotic treatment?
- What is the mechanism by which silver resistance also confers increased resistance to antibiotics? *i.e.* why do the *cysD* and *ycdB* expressing strains, which have lowered production of ROS, have increased MIC for ampicillin and kanamycin? Do these findings support a role for a radical-mediated action of antibiotics, a very topical and much-debated issue? What about resistance of strains to other antibiotics?
- If increased resistance to silver in strains harbouring *ycdB* and *cysD* plasmids led to increased resistance to antibiotics, why did mutants of *yeaO* and *ydgT*, which conferred increased resistance to silver compared to the wildtype, not lead also to increased antibiotic resistance?
- Do the mechanisms of silver resistance described here bear any resemblance to potential resistance outside of laboratory settings, for example in clinical settings where silver is used for antimicrobial purposes? Could mechanisms of resistance discovered in the laboratory, such as the ones described here, be engineered for the purposes of bioremediation, for example?

In summary, the work described offers a novel approach, namely exploration of bacterial resistance to silver in a laboratory setting, that sheds new light on the mechanisms of silver antimicrobial action. This research contributes to fundamental aspects of the biochemistry and evolution of bacteria and, finally, provides glimpses of possible future practical applications, for example in antibiotic research, in the biocatalytic synthesis of nanoparticles or in bioremediation.

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