Silver potentiates aminoglycoside toxicity by enhancing their uptake

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Summary

The predicted shortage in new antibiotics has prompted research for chemicals that could act as adjuvant and enhance efficacy of available antibiotics. In this study, we tested the effects of combining metals with aminoglycosides on *Escherichia coli* survival. The best synergising combination resulted from mixing aminoglycosides with silver. Using genetic and aminoglycoside uptake assays, we showed that silver potentiates aminoglycoside action in by-passing the PMF-dependent step, but depended upon protein translation. We showed that oxidative stress or Fe-S cluster destabilization were not mandatory factors for silver potentiating action. Last, we showed that silver allows aminoglycosides to kill an *E. coli* gentamicin resistant mutant as well as the highly recalcitrant anaerobic pathogen *Clostridium difficile*. Overall this study delineates the molecular basis of silver's potentiating action on aminoglycoside toxicity and shows that use of metals might offer solutions for battling against increased bacterial resistance to antibiotics.

Introduction

Increasing bacterial resistance to antibiotics is a major health issue, particularly in the context of the low discovery rate for new antibiotics (Nordmann *et al.*, 2007). A solution to this problem is to use antibiotic-potentiating or adjuvant agents to improve the efficacy of existing antibiotics. Augmentin that combines a β-lactam and a β-lactamase inhibitor is a well-known example of an antibiotic-adjuvant association, which has been used extensively to treat infections caused by β-lactam resistant bacteria (Leigh *et al.*, 1981; Worthington and Melander, 2013). Other types of potentiating effects can be obtained by facilitating the entry of antibiotics into target bacterial cells (Mislin and Schalk, 2014).

Aminoglycosides are bactericidal antibiotics, which target ribosomes. They comprise kanamycin, tobramycin, gentamicin (Gm), and streptomycin, and are commonly used worldwide, thanks to their high efficacy and low cost (Davis, 1987). One limitation of these antibiotics is their ineffectiveness against anaerobic bacteria. Recently, we showed that the efficacy of aminoglycosides could be lowered by reduced iron bioavailability, which reduced Gm uptake (Ezraty *et al.*, 2013; Ezraty and Barras, 2016). Uptake of aminoglycosides in Gram-negative bacteria is a three-step process (Taber *et al.*, 1987). Aminoglycosides, which are strongly cationic drugs at physiological pH, first bind electrostatically to the negatively charged outer membrane and then reach the periplasm through porin channels. Uptake across the inner membrane is an energy-dependent process that can be described in two sub-steps forming a feed forward loop. Energy dependent phase I (EDP-I) requires the proton motive force (PMF), which allows aminoglycoside to reach the cytosol via an electrophoresis-like process. Eventually aminoglycosides act on translating ribosomes. Energy dependent phase II (EDP-II) is when inhibited ribosomes release misfolded aborted hydrophobic proteins, which incorporate into, and disturb membrane organization. The consequence is a massive uptake of aminoglycosides (Hurwitz *et al.*, 1981).

Respiratory complexes I and II, which are the major sources of PMF, contain numerous Fe-S clusters, which are essential for their activity. Biogenesis of Fe-S cluster is carried out either by the ISC or by the SUF system. ISC is used under normal growth conditions, whereas SUF is used under stress conditions, such as iron limitation. For reasons that remain unclear, the SUF system is much less efficient in targeting Fe-S clusters into complexes I and II. The consequence is that complexes I and II are less active. Thus, under iron limitation, low efficiency of maturation of complexes I and II by SUF leads to lowered PMF, reduced EDP-I controlled aminoglycoside uptake, and enhanced aminoglycoside resistance. Interestingly, a low level of PMF could also account for the high resistance of

anaerobic bacteria to aminoglycosides as they use fermentative metabolism. Another connection between aminoglycosides and metals was highlighted by a previous study that attributed an aminoglycoside-potentiating effect to silver. It was proposed that silver enhanced reactive oxygen species (ROS) production, which contributed to the silver potentiating effect on Gm toxicity (Morones-Ramirez *et al.*, 2013).

In this study, we screened a series of metals for their influence on the susceptibility of *E. coli* to aminoglycosides. Silver, and to some extent copper, stood out as potentiating aminoglycoside efficacy. We reinvestigated the molecular basis of the silver potentiating effect and concluded that it is (i) independent of PMF and allows by-passing EDP-I, (ii) dependent on protein translation, and (iii) independent of ROS production. Finally we showed that silver could be used to combat highly natural aminoglycoside resistant bacteria, such as the Gram-positive anaerobic pathogen, *Clostridium difficile*.

Results

Assessing the potentiating effects of metals on Gm toxicity

In LB and under aerobic conditions, *E. coli* exhibited a minimal inhibitory concentration (MIC) value to Gm (Gm) of 2.8 +/- 0.2 µg/ml (Fig. 1A). This value was not modified upon addition of sub-lethal concentrations of iron, cobalt, zinc or nickel-containing solutions (100 µM, see Table S1) (Fig. 1A). In contrast, addition of silver (8 µM) led to a 10 fold reduction of the MIC value (0.17 +/- 0.1 µg/ml) (Fig. 1A). A dose-response effect of silver was observed (Fig. S1A). Addition of copper (100 µM) produced also a small but significant decrease of the MIC value, i.e. 2 +/- 0.4 µg/ml (Fig. 1A). In M9 minimal medium, the MIC value to Gm decreased to 0.25 +/- 0.02 µg/ml and was influenced neither by the addition of sub-lethal concentration of silver (4 nM see Table S1) nor by that of copper (0.25 µM see Table S1) (Fig. S1B). In M9 minimal medium supplemented with 0.2% Casamino acids, *E. coli* exhibited a MIC value to Gm of 0.5 +/- 0.06 µg/ml, addition of silver (50 or 100 nM) produced a small but significant 2 fold decrease of the MIC value. However, survival rate of *E. coli* to Gm treatment in M9 minimal medium supplemented with 0.2% Casamino acids was not modified by the presence of silver (50 or 250 nM) (Fig. S1D). In Neidhardt's low phosphate minimal medium, *E. coli* exhibited a MIC value to Gm of 0.2 +/- 0.02 µg/ml, which was not modified by the addition of sub-lethal concentration of silver (0.01 µM see Table S1) (Fig. S1E). Consistently, survival rate of *E. coli* to Gm treatment in Neidhardt's low phosphate minimal medium was not modified by the presence of silver (Fig. S1F). Altogether these studies highlighted the importance of the growth conditions, which can modify both the MIC values of Gm and the capacity of metals to enhance the toxicity of Gm. The reason why silver seems to potentiate Gm toxicity only in LB grown *E. coli* is unknown and this issue is discussed later in the manuscript. A thorough comparative study between media was out of the scope of the present study and we used LB throughout all next experiments.

Silver potentiates the activity of all aminoglycosides in *E. coli*

We tested the potentiating activity of silver on an extended set of antibiotics from diverse families. Silver was found to lower more than 10 fold the MIC values for all three bactericidal aminoglycosides tested, i.e. kanamycin, tobramycin and streptomycin. A reduction of only 2 fold was observed with spectinomycin, an aminoglycoside that is bacteriostatic (Fig. 1B). Similarly, a slight potentiating effect (less than 20% decrease of the MIC value) was

observed when used in conjunction with nalidixic acid and norfloxacin but not ciprofloxacin, although all three belong to the quinolone family (Fig. 1B). Modest reduction of the MIC values was also noted when silver was used in conjunction with ampicillin, tetracycline or chloramphenicol (Fig. 1B). Collectively, these results showed that silver exerts a potentiating effect mostly on aminoglycosides.

Silver enhances Gm toxicity by acting independently of PMF

Gm penetrates across the cytoplasmic membrane via a PMF-dependent process. We therefore wondered whether PMF was required for the silver potentiating effect. To test this, we used pharmacological and genetic approaches. Carbonyl cyanide-m-chlorophenylhydrazone (CCCP), an uncoupler H^+ ionophore, was used to dissipate the PMF. Then, we followed the survival rate of *E. coli* after 1.5 hours of Gm treatment, in absence or in presence of CCCP and with or without silver. As previously reported by us and others (Ezraty *et al.*, 2013; Allison *et al.*, 2011), the presence of CCCP enhanced resistance of *E. coli* to Gm-mediated killing, because it abolishes PMF. In contrast, adding silver reversed the effect and re-sensitized *E. coli* even in the presence of CCCP (Fig. 2A). We also tested mutants known to have low levels of PMF (Ezraty *et al.*, 2013). Specifically, we tested either a strain deleted of *nuo* and *sdh* operons, which lacks complexes I and II, or a strain deleted of *iscUA* genes, which poorly matures complexes I and II. Survival tests showed that Δ*nuo* Δ*sdh* and Δ*iscUA* strains exhibited enhanced resistance to Gm (Fig. 2A). Consistently, MIC values of both Δ*nuo* Δ*sdh* (3.6 +/- 0.3) and Δ*iscUA* (6.1 +/- 0.5) strains were higher than wt (Fig. 2B). Adding silver cancelled differences between mutant and wt strains. All three strains, wt, Δ*nuo* Δ*sdh* and Δ*iscUA*, showed the same survival loss of about 5 log in 1.5 hours (Fig. 2A) and all three exhibited the same MIC value, i.e. 0.17 +/- 0.1 µg/ml (Fig. 2B). Taken together, both the pharmacological and genetic approaches demonstrated that silver enhanced Gm toxicity in a PMF-independent manner.

Silver restores Gm uptake in a PMF-deficient strain

We then tested whether the potentiating effect of silver was due to an increased uptake of Gm. The intracellular accumulation of Gm was monitored over time by using tritiated Gm (^{3}H-Gm). As previously reported, both the Δ*nuo* Δ*sdh* and Δ*iscUA* strains were drastically altered in Gm uptake (Fig. 2C) (Ezraty *et al.*, 2013). In contrast, addition of silver restored Gm uptake in both mutants (Fig. 2C). Uptake in the Δ*nuo* Δ*sdh* mutant was more efficient as

compared with both the wt and the Δ*iscUA* mutant; we have no explanation for this phenomenon. Moreover, in wt strain, silver allowed uptake of Gm applied at sub-lethal concentration (Fig. 2D). These results showed the potentiating effect of silver to be due to its capacity to enhance uptake of Gm. Taken together with the results above, these data demonstrated that silver by-passes the EDP-I PMF-dependent step of the Gm entry process.

Silver potentiating effect is EPD-II dependent

The EDP-II is protein translation-dependent. This is shown by the fact that chloramphenicol (Cm) a bacteriostatic antibiotic inhibiting translation, has a protective effect against Gm (Fig. 3). Therefore we asked whether Cm could interfere with the silver potentiating effect. Survival rate of *E. coli* to Gm treatment was the same whether silver was added or not to Cm-treated cells (Fig. 3). This result showed that silver needs the EDP-II, i.e. protein translation, to potentiate Gm toxicity.

ROS production does not explain the positive combination between silver and aminoglycosides

Silver is known to destabilize Fe-S clusters (Liu and Imlay, 2013). Morones-Ramirez *et al.* attributed potentiating effect of silver on aminoglycoside activity to enhanced production of harmful ROS following Fe-S destabilization by silver, release of free iron and Fenton chemistry (Morones-Ramirez *et al.,* 2013). To further investigate this issue, we tested the silver-potentiating effect of Gm on anaerobically grown cultures. An *E. coli* strain grown in anaerobic fermentative conditions exhibited enhanced resistance to Gm (Fig. 4A). However, adding silver to anaerobically growing *E. coli* allowed Gm to cause a loss of 4 logs in survival (Fig. 4A). This did not support the hypothesis of a contribution of ROS. We tested the role of ROS in silver potentiating effect by using *E. coli* strains altered in their redox homeostasis control. ROS-hyper-sensitive strains lacking superoxide dismutases (Δ*sodA* Δ*sodB*) or the H_2O_2-stress responding master regulator (Δ*oxyR*) exhibited similar sensitivity to silver potentiating effect as compared with the wild type parental strain (Fig. 4B, S2). Similarly, a strain expressing constitutively the H_2O_2-stress responding regulon (*oxyRc*) exhibited a wild type-like sensitivity to the silver-potentiating effect of Gm (Fig. 4B, S2). Altogether, these observations indicate that ROS do not play a major role in the Gm-potentiating activity of silver.

Fe/S clusters destabilization is not mandatory for silver potentiating action.

An *iscS* mutant exhibits enhanced resistance to silver (Fig S3A) (Morones-Ramirez *et al.,* 2013). A possibility put forward by others is that the *iscS* enhanced resistance was due to a reduced level of Fe-S cluster biogenesis, hence a reduced possibility of fueling Fenton chemistry upon Fe-S cluster destabilization (Morones-Ramirez *et al.,* 2013). However, iscS mutation is pleiotropic and it is unsafe to interpret its phenotype as being the sole consequence of an alteration of Fe-S cluster biogenesis. Therefore, we tested an *iscUA* mutant, which is specifically altered in Fe-S cluster biogenesis. The *iscUA* mutant was hyper-sensitive to silver (Fig. S3B). The reason for this is probably because silver targets Fe-S clusters in key enzymes, whose decreased activities are detrimental for fitness, as this has already been proposed (Xu and Imlay, 2012). In any case, the phenotype of *iscUA* mutant shows that decrease in Fe-S cluster capacity sensitizes cells to silver toxicity, rather than protects it.

Then the question arose of why an *iscS* mutant still exhibits enhanced resistance to silver. IscS degrades L-cysteine and L-cysteine is known like histidine to help *E. coli* to resist to silver by sequestering it (Fig. S3C) (Matsumura *et al.*, 2003). Hence a possibility was that the *iscS* mutant showed enhanced resistance to silver because it accumulated a higher concentration of cysteine. To test this hypothesis, we over-produced CsdAE, a highly efficient hetero-dimeric cysteine desulphurase, in an *iscS* mutant and observed that this did suppress silver resistance of the *iscS* strain. As a control, we showed that a strain synthesizing a mutated inactive version of CsdA was unable to act as a suppressor of the *iscS* resistance (Fig. S3D). The data above suggest that Cys accumulation is the reason for the hyper-resistance to silver of *iscS* mutant.

Silver sensitizes *E. coli* Gm^R strain and aminoglycoside resistant pathogens bacteria

The *aaC1* gene encodes a N-acetyltransferase that modifies and inactivates Gm. Accordingly, an *E. coli aac1*+ strain (Gm^R) exhibited a MIC value of 121+/-6 µg/ml, i.e. ca. 50 fold higher than wt and remained partially viable in the presence of 100 µg/ml Gm (Fig. 5A and B). Uptake experiments revealed that *E. coli aac1*+ strain failed to accumulate intracellular Gm (Fig. 5C). Remarkably, adding silver reduced the MIC value to $4.3_{+/-1}$, and provoked bacteriostatic and bactericidal effects with 5 and 100 µg/ml Gm treatment, respectively (Fig. 5A and B). Moreover, Gm uptake tests showed that silver allowed both Gm incorporation and accumulation to resume in the Gm^R strain (Fig. 5C). Taken together, these results showed that silver is able to sensitize a Gm^R strain to Gm treatment.

Clostridium difficile, an anaerobic enteropathogen causing infectious diarrhea, is well known for its aminoglycoside resistance (Båverud *et al.*, 2004). Indeed, MIC values of *C. difficile* for Gm, tobramycin and kanamycin were found to be very high (Fig. 5D). The question of the silver potentiating capacity was therefore worth exploring. Spectacularly, in the presence of silver, the MIC values of *C. difficile* dropped down from $43_{+/-4}$ to $13_{+/-3}$ µg/ml for Gm, from $115_{+/-8}$ to $15_{+/-4}$ µg/ml for tobramycin and from $313_{+/-26}$ to $13_{+/-4}$ µg/ml for kanamycin (Fig. 5D). Thus, overall treatment with silver decreased the MIC values for aminoglycosides from 3- to 24-fold. Taken together, these results expanded the spectrum of silver potentiating effect to a recalcitrant Gram-positive pathogen.

Discussion

Screening of different metals allowed us to identify silver as an enhancer of aminoglycoside treatment efficacy against *E. coli*. Moreover, we showed that silver allowed sensitizing the Gram-positive aminoglycoside resistant enteropathogen *C. difficile* to Gm, tobramycin or kanamycin treatments. The potentiating effect of silver has been reported before by Morones-Ramirez *et al.*, (2013), but we offer a different explanation regarding the underlying mechanism. In particular we rule out a role for ROS in this process as the enhancing effect was also observed to arise under anaerobiosis and in mutants altered in anti-ROS activities. Moreover, we show that silver enhances Gm uptake in a PMF-independent, protein translation-dependent way.

Silver has been used as an antiseptic for centuries. Here we investigated the molecular basis of both its toxicity and its adjuvant activity. We found an *iscUA* mutant, specifically altered in Fe-S biogenesis, to be highly sensitive to silver. The simplest interpretation is that Fe-S clusters are targeted by silver ions, due to their thiophilicity (Xu and Imlay, 2012). One can wonder why the back-up stress responding Fe-S cluster biogenesis system, SUF, does not take over the absence of ISC in the *iscUA* mutant. Presumably, at the silver concentration used, the SUF system is not sufficient either to keep up with the overall cellular demand in the presence of silver or to target clusters efficiently enough to essential enzymes such as IspGH. In both cases, bacterial viability is compromised.

Silver potentiating effect was observed when *E. coli* was grown in LB but was not observed when *E. coli* was grown in minimal medium, M9 or Neidhardt's low Pi medium. The influence of medium is best illustrated by the fact that the MIC values for Gm are 10 fold lower in minimal medium than in LB. One possibility was that silver damaged Fe-S bound dehydratase enzymes required for branched-chain amino acid synthesis. If so translation could have been arrested, hence preventing silver to potentiate Gm toxicity as we showed its effect to be translation-dependent. This was investigated by asking whether the addition of Casamino acids could allow silver potentiating effect to resume even in M9 medium. Results were ambiguous as some potentiating effect was observed using MIC values but it was much less than in LB (i.e. 2 fold in minimal medium supplemented with Casamino acids vs. 10 fold in LB). Moreover, survival tests failed to reveal a silver potentiating effect. Therefore, for now we have no clear-cut explanation to offer for the medium dependency. It could be due to metabolic changes in different media, differences in pH and possible consequences on ΔpH trans-membrane component of the PMF, sequestering of Gm by LB contained components, occurrence of phosphate silver complexes, or a combination of these different effects. A dedicated systematic study will need to be conducted to solve this issue.

Silver potentiating effect of Gm was observed under anaerobiosis. Moreover, we failed to observe a correlation between anti-ROS capacity of the cell and its sensitivity to a mix of silver and Gm. Altogether, this strongly suggested that ROS has no major role in in silver potentiating effect.

Our characterization of the silver-potentiating effect demonstrated that it was independent of PMF, as it restored Gm sensitivity of strains with reduced PMF levels such as Δ*nuo* Δ*sdh* or Δ*iscUA* mutants. A possibility is that silver destabilizes the membrane allowing Gm to get access to the cytosol more easily. In support of this view, we might cite electron microscopy studies, which showed that silver perturbs cell wall perturbation after silver treatment (Feng *et al.*, 2000) and a recent system-based analysis of silver stress pointed out potential dysregulation of fatty acid homeostasis (Saulou-Bérion *et al.*, 2015). However, we also showed that silver-potentiating effect of Gm toxicity remained dependent on translation. This implies that the membrane disturbance induced by silver is not sufficient for massive Gm uptake and needs additional contribution from mis-localised aborted polypeptides, predicted to occur during EDP-II. Alternatively, silver might act directly on ribosomes, and might allow released of mis-folded aborted polypeptides that would eventually go to the membrane and allow massive Gm uptake. In this hypothesis, silver would cause an EDP-II like step. Further studies will be required for establishing the actual mechanism of action of silver. Under some aspects, it seems similar to toluene and polymixin B (Davis, 1960). In contrast, it differs from the mode of action of others chemicals recently reported to potentiate Gm toxicity. For instance, mannitol and fructose led to the generation of PMF via increased NADH production, hence facilitating the EDP-I step (Allison *et al.*, 2011). Another study showed that non-buffered L-arginine, by impacting the ΔpH transmembrane component of the PMF, increased susceptibility to Gm (Lebeaux *et al.*, 2014).

Strict anaerobes are unable to efficiently take up aminoglycosides and therefore are resistant to this family of antibiotics (Schlessinger, 1988) as observed for *C. difficile* (Båverud *et al.*, 2004). However, it is important to stress that ribosomes of clostridia can be bound and inhibited by aminoglycosides (Bryan *et al.*, 1979). Our study points to a new approach to try to fight vegetative cells of this bacterium, as silver was found to overcome the probable natural lack of permeability of their membrane for aminoglycoside. This is an important result as *C. difficile* infection is the most common cause of identifiable diarrhea in hospitalized adult patients and the incidence and severity of *C. difficile* infections increased (Lessa *et al.*, 2015 ; Rupnik *et al.*, 2009). In conclusion, our study depicts a new molecular mechanism for the positive potentiating effect of silver on aminoglycoside activity, and shows

that use of metals might offer solutions for battling against increased bacterial resistance to antibiotics. It is noteworthy to remind that copper was also proposed for boosting various drug treatments (Speer *et al.*, 2013). Moreover, our study opens the way for fighting natural aminoglycoside resistant anaerobic pathogens.

Experimental procedures

General microbiology and molecular biology

In this study, we used *E. coli* K-12 derivatives strains and *C. difficile* 630 Δ*erm* (Hussain *et al.*, 2005) (for details see Table). The LB medium used in this study comes from Difco. The phosphate limiting minimal medium was the MOPS (pK_a 7.2) medium described by Neidhardt contained 0.1 mM K_2HPO_4 plus 9.8 mM KCl and 0.2 % glucose (Neidhardt *et al.*, 1974). P1*vir* transduction was used to move alleles into a different background. pCsdAE^{C358A} was constructed by site-directed mutagenesis using pCsdAE plasmid as template (Trotter *et al.*, 2009), the oligonucleotides csdAE_C358A_F (CGGGCCGGGCAGCATGCCGCTCAGCCGCTAC) and csdAE_C358A_R (GTAGCGGCTGAGCGGCATGCTGCCCGGCCCG), *Pfu* Turbo DNA polymerase (Invitrogen) and *DpnI* from NEB. All plasmids were sequenced (Beckman Coulter Genomics) for verification.

Strains	Relevant Genotype	Source or Reference
E. coli K-12 strains		
MG1655	Parental strain	Lab collection
DV597	MG1655 Δ*iscUA*::Cm^r	(Vinella *et al.*, 2009)
LL111	MG1655 Δ*iscS*::Cm^r	(Trotter *et al.*, 2009)
BEFB20	MG1655 Δ*sdhB* Δ*nuo*::*nptI* Kan^r	(Ezraty *et al.*, 2013)
BEFB10	MG1655 *trp*::Hfr::Gm^r	(Ezraty *et al.*, 2013)
BEFB01	MG1655 Δ*sodA*::Cm^r Δ*sodB*::Kan^r	(Ezraty *et al.*, 2013)
BEFB02	MG1655 Δ*oxyR*::Cm^r	(Ezraty *et al.*, 2013)
QC2433	MG1655 *oxyR*c	Lab collection
C. difficile		
630Δ*erm*		(Hussain *et al.*, 2005)
Plasmids		
pCsdAE		Lab collection (Trotter *et al.*, 2009)
pCsdAE^{C358A}		This study

MIC determination

The drugs tested were dissolved in LB medium for *E. coli* tests and in water for *C. difficile* tests. The range of concentrations of each drug was twice the MIC. For *E. coli*, one hundred microliters (100µL) of each 2X concentration tested were added in a 96-well microplate. Each well was inoculated with 100µL of a fresh LB bacterial inoculum of 2 x 10^5 CFU/mL and the plates were incubated at 37°C for 18 hours under aerobic conditions and agitation 150 rpm. *C. difficile* strain 630Δ*erm* was grown anaerobically (5 % H_2, 5 % CO_2, and 90 % N_2) in microplate. Each well was inoculated with 1 mL of a fresh BHI bacterial inoculum at an OD_{600} of 0.01 and the drug was added at different concentrations in the presence or absence of $AgNO_3$ at 20 µM. The plates were incubated at 37°C for 20 h under anaerobic conditions. MIC was defined as the lowest drug concentration that exhibited complete inhibition of microbial growth.

Lethality studies on LB agar plates

Strains were grown aerobically (shaking 150 rpm) in minimal media M9 at 37°C. When the OD_{600} reached 0.2, serial dilutions of cell suspensions in phosphate buffer (0.05 M, pH 7.4) were spread onto M9 media agar plates with different concentrations of silver nitrate and cysteine (specified in each figures). C.f.u. were counted after incubation for 16 h at 37°C.

Metals

For synergetic effect with antibiotics $AgNO_3$ was used at 8 µM for *E. coli*, unless noted otherwise, and 20 µM for *C. difficile*, $FeSO_4$, $CoCl_2$, $Cu_2(OAc)_4$, $Zn(OOCCH_3)_2$ and $NiCl_2$ were used at 0.1 mM.

Survival rate

Overnight cultures (16 h) were diluted 100 times and grown aerobically in LB at 37°C to an OD_{600} of 0.2. At this point, antibiotics and/or silver were added to the cells and after 1.5 h cells were taken, diluted in PBS buffer, spotted on LB agar and incubated at 37°C for 16 h. Cell survival was determined by counting colony-forming units per ml (c.f.u./ml). The absolute c.f.u at time-point 0 (used as the 100 %) was ~5x10^7 cells/ml in all experiments. When used, the PMF uncoupler, carbonyl cyanide-m-chlorophenylhydrazone (CCCP) was added at a final concentration of 1 µg/ml. When used, chloramphenicol (Cm) was added 30 min before Gm treatment at a final concentration of 200 µg/ml. The Cm stock solution was extemporaneously prepared at 20 mg/ml with 1/3 ethanol and 2/3 H_2O. Synergic effect in anaerobic conditions was performed as previously described but in

anaerobic chamber (Coy Chamber) with anaerobic media (previously equilibrated in the anaerobic chamber for 24 h).

For silver sensitivity experiments M9 minimal media was used instead LB in order to minimized the variability of the results due to the chelation of the metal by medium components present in the LB (Feng *et al.*, 2000). Overnight cultures (16 h) were diluted 100 times and grown aerobically in minimal media M9-0.2 % glycerol at 37ºC to an OD_{600} of 0.2. At this point, $AgNO_3$ was added to the cells and after different incubation times (specified on each figure) cells were taken, diluted in phosphate buffer (0.05 M, pH 7.4), spotted on LB agar and incubated at 37°C for 16 h.

Gm uptake

[^{3}H]-Gm (20 µCi/mg; Hartmann Analytic Corp.) was added to a final concentration of 5 or 0.25 µg/ml and cultures were incubated at 37°C on a rotary shaker. At given times, 500 µl aliquots were removed and collected on a 0.45 µm-pore-size HAWP membrane filter (Millipore) pretreated with 1 ml of unlabeled Gm (250 µg/ml). Filters were subsequently washed with 10 ml of 3 % NaCl, placed into counting vials, dried for 30 min at 52°C whereafter 8 ml of scintillation liquid were added and incubated overnight at room temperature. Vials were counted for 5 min. Gm uptake efficiency is expressed as total accumulation of Gm (nanograms) per 10^8 cells.

Statistical analysis

Mann-Whitney U tests were performed using the QI-Macros software (KnowWare International, Inc., Denver, CO)

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Legends:

Figure 1. Silver potentiates aminoglycosides in *E. coli*

(A) Minimal Inhibitory Concentration (MIC) of Gm (in μg/ml) in the presence of different metals was calculated. *E. coli* strain MG1655 was grown in LB under aerobic conditions in presence of 8 μM $AgNO_3$, or 0.1 mM $FeSO_4$, $CoCl_2$, $Cu_2(OAc)_4$, $Zn(OOCCH_3)_2$ and $NiCl_2$. MIC values were calculated as described in Materials and Methods. (B) MIC values of *E. coli* MG1655 for different antibiotics as indicated on top of the graphs. $AgNO_3$ concentration used was 8 μM. *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001 (Mann-Whitney U test).

Figure 2. Silver potentiates Gm activity against *E. coli* by a PMF-independent uptake

(A) Percentage survival of *E. co*li MG1655 after 1.5 h treatment with Gm (5 μg/ml) and/or $AgNO_3$ (8 μM). Genotypes of strains studied (wt, Δ*nuo* Δ*sdh* and Δ*iscUA*) are indicated on top of the graph. Wt strain was also studied after growth in the presence of CCCP (1 μg/ml) as indicated. Black, dark grey, light grey and red bars are for untreated, $AgNO_3$, Gm and Gm and $AgNO_3$ together. (B) MIC values of wt, Δ*iscUA* and Δ*nuo* Δ*sdh* strains grown in the presence of Gm (dark bar) or Gm and $AgNO_3$ (8 μM) (red bar). (C) Presence of silver restore tritiated Gm (^{3}H-Gm) uptake in Δ*iscUA* and Δ*nuo* Δ*sdh* mutants. Uptake was measured by incubating early exponential-phase cultures (OD_{600} ~ 0.2 with 5 μg/ml ^{3}H-Gm in presence or absence of $AgNO_3$ (8 μM) at 37°C. (D) Gm uptake test with sub-lethal Gm concentration. The *wt* strain was grown up to OD_{600} ~ 0.2 with 0.25 μg/ml ^{3}H-Gm in the presence or absence of $AgNO_3$ (8 μM) at 37°C. *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001 (Mann-Whitney U test).

Figure 3. Silver potentiates Gm activity against *E. coli* by a translation-dependent process

Percentage survival of *E. co*li MG1655 after 1.5 h treatment with Gm (5 μg/ml) and/or $AgNO_3$ (8 μM) with or without chloramphenicol (Cm). Strain was grown in the presence of saturating concentration of Cm (200 μg/ml) for 30 min before adding Gm and/or $AgNO_3$. Black, dark grey, light grey and red bars represent untreated, $AgNO_3$-treated, Gm-treated and Gm + $AgNO_3$-treated cultures.

Figure 4. ROS production does not explain the positive combination between silver and aminoglycoside

(A) Percentage survival of an *E. coli* MG1655 after 1.5 h treatment with $AgNO_3$ (dark grey bar) Gm (light grey bar), Gm + $AgNO_3$ (red bar) in anaerobic condition. Gm was used at 5 µg/ml and silver at 8 µM. Anaerobic condition was realized in anaerobic chamber (Coy Chamber; 5 % H_2, and 90 % N_2) with anaerobic LB media previously equilibrated in the anaerobic chamber for 24 h. (B) Percentage survival of *E. co*li MG1655 after 1.5 h treatment with $AgNO_3$ (dark grey bar) Gm (5 µg/ml) (light grey bar), Gm + $AgNO_3$ (2 µM) (blue bar), Gm + $AgNO_3$ (4 µM) (yellow bar), Gm + $AgNO_3$ (6 µM) (green bar), Gm + $AgNO_3$ (8 µM) (red bar) in aerobic condition. Genotypes of strains studied (wt, Δ*sodA* Δ*sodB*, Δ*oxyR* and *oxyR*^{C}**)** are indicated on top of the graphs. *P ≤ 0.05 and **P ≤ 0.01 (Mann- Whitney U test).

Figure 5. Silver sensitizes Gm resistant strain of *E. coli* and *C. difficile*

(A) MIC values (µg/ml) of an *E. coli* Gm^R mutant (*aac1*+ strain) grown in presence of $AgNO_3$ (8 µM). (B) Percentage survival of an *E. coli* Gm^R mutant after 1.5 h treatment with $AgNO_3$ (dark grey bar) Gm (light grey bar), or Gm and silver together (red bar). Gm was used at 5 or 100 µg/ml, as indicated, and $AgNO_3$ 8 µM. (C) Gm uptake test. The Gm^R mutant was grown up to OD_{600} ~ 0.2 with 5 µg/ml ^{3}H-Gm in the presence or absence of $AgNO_3$ (8 µM) at 37°C. (D) MIC in µg/ml of *C. difficile* strain 630Δ*erm* was calculated for different antibiotics. Strain 630Δ*erm* inoculated at an OD_{600} of 0.01 was grown for 20 hours in the presence or absence of 20 µM $AgNO_3$ and in the presence of increasing concentrations of Gm, tobramycin and kanamycin. After 20 h of incubation at 37°C, the OD_{600} of the strain 630Δ*erm* grown in the absence of antibiotics was similar in the presence or absence of 20 µM. *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001 (Mann-Whitney U test).

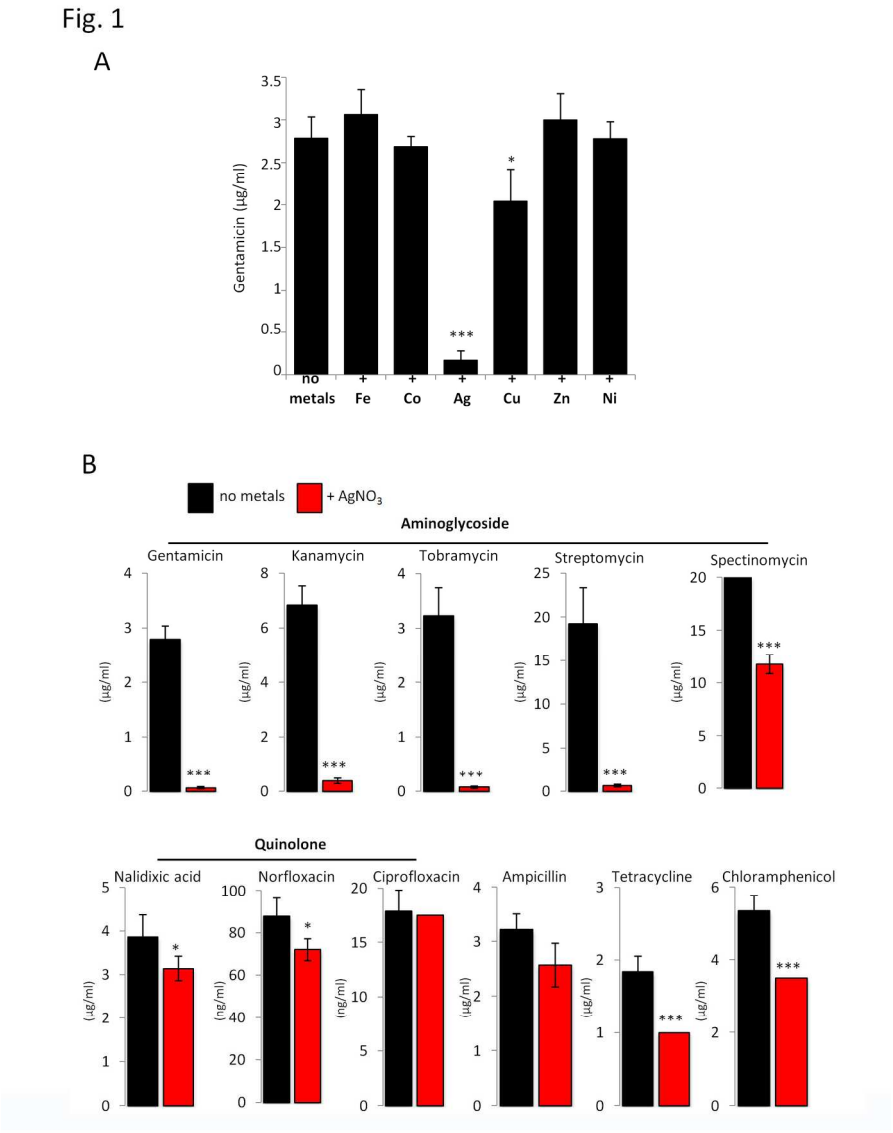

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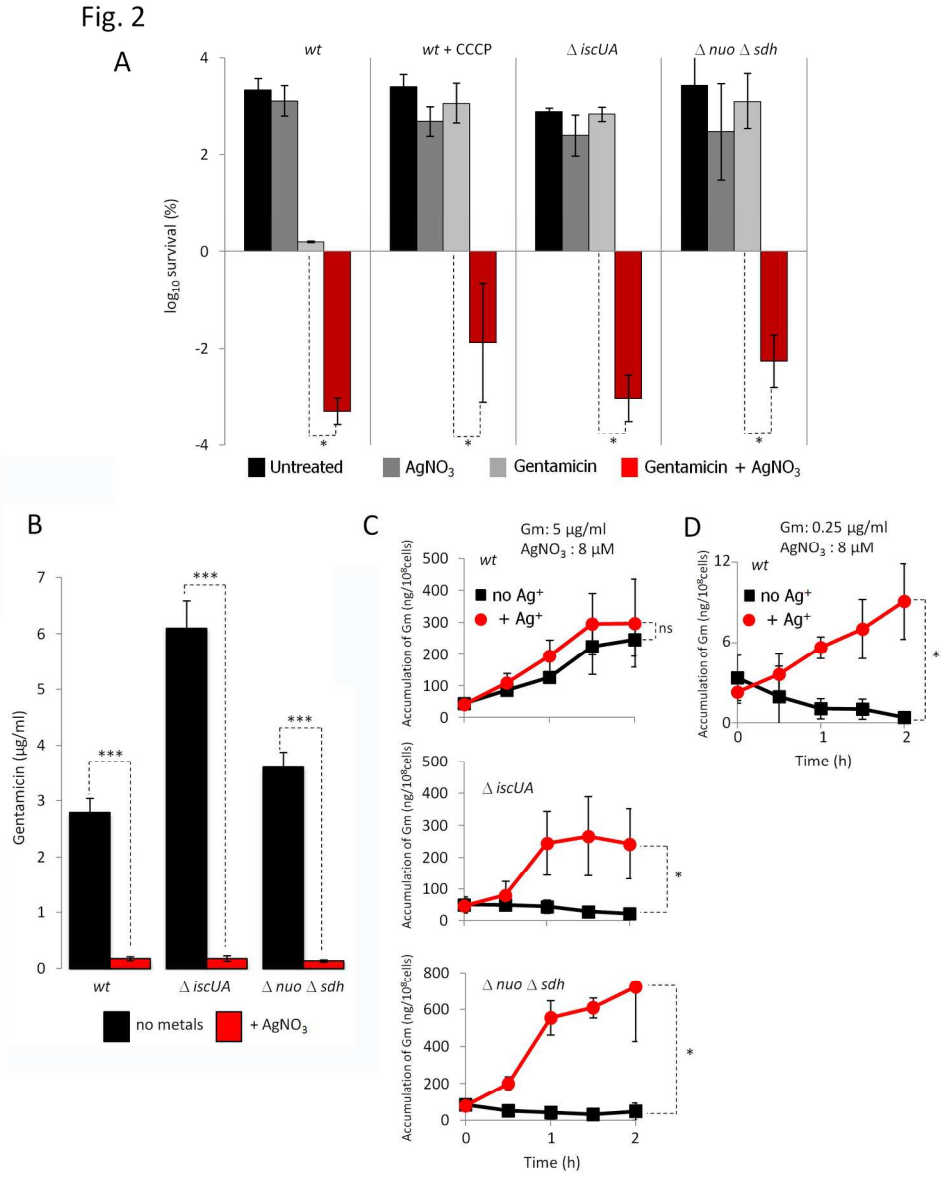
Fig. 2



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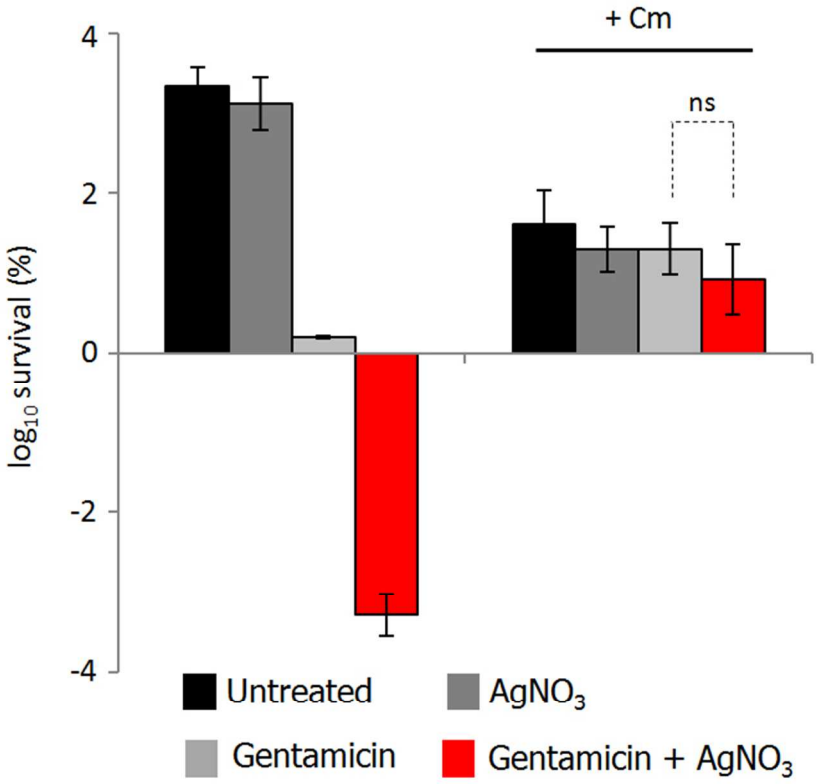

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Fig. 4

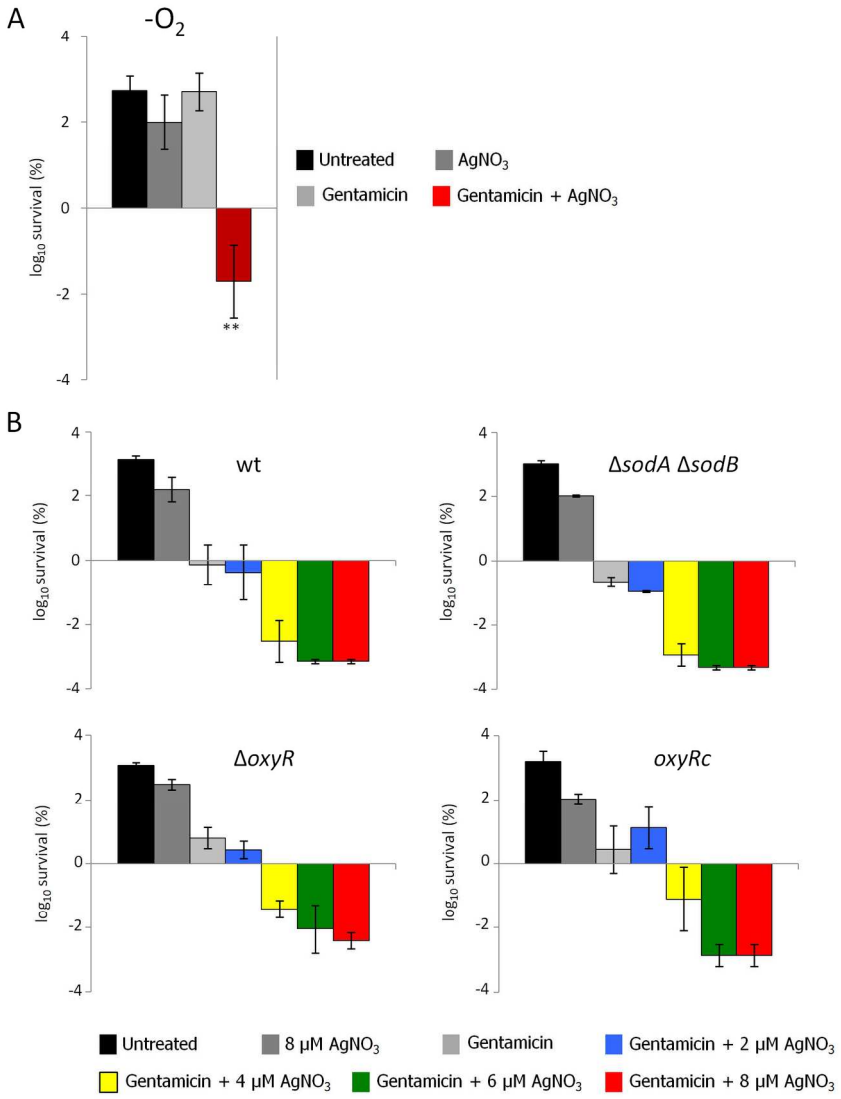

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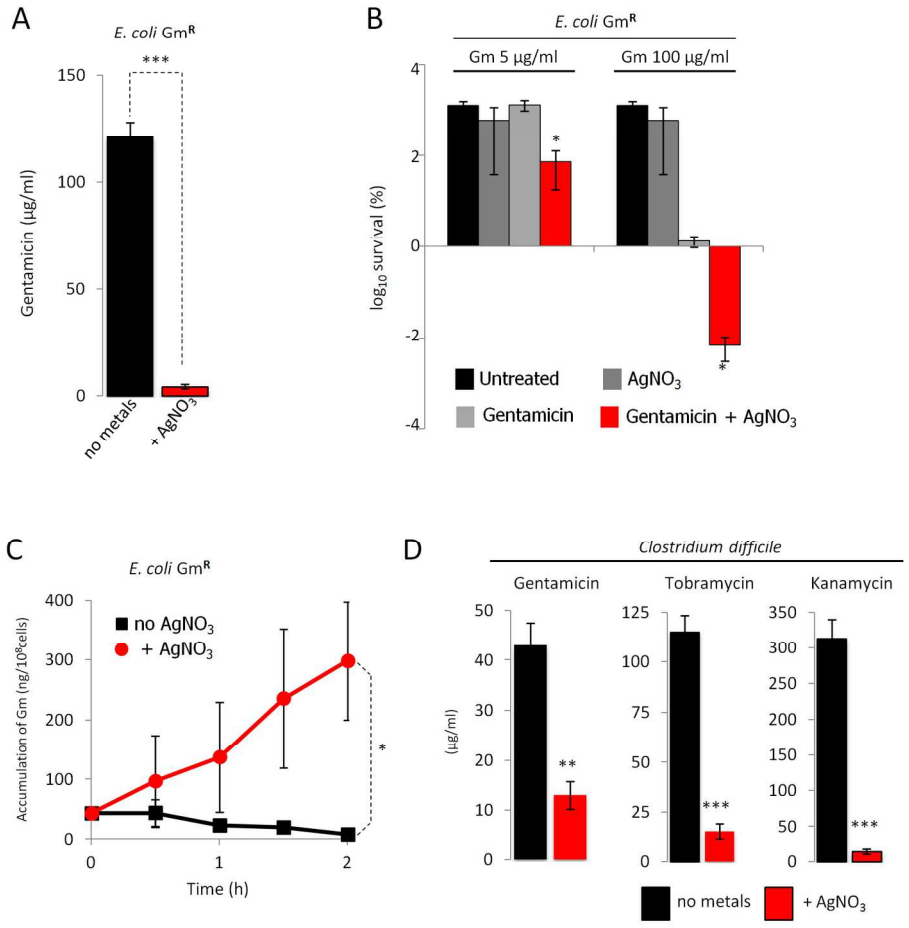
Fig. 5



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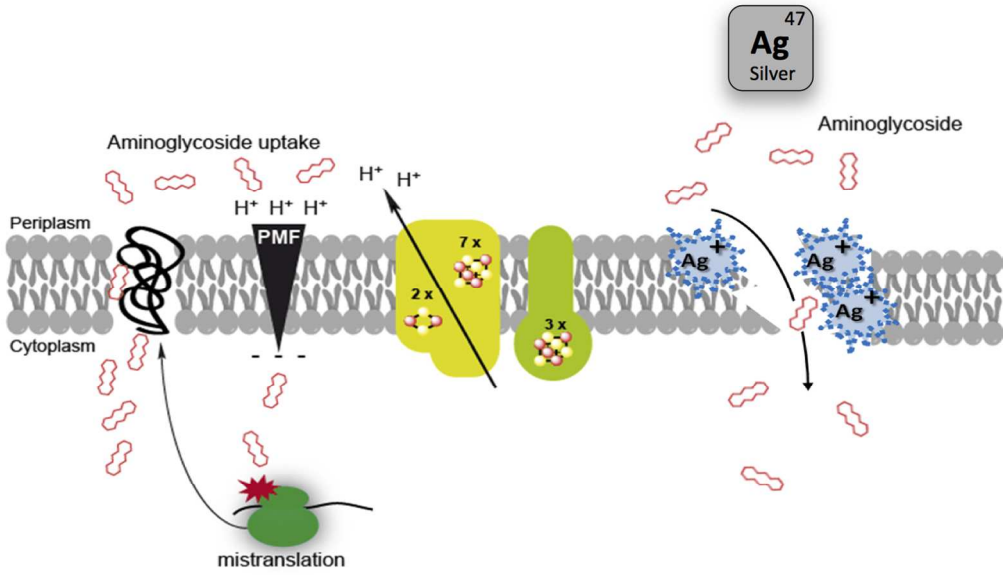

Silver potentiates aminoglycoside efficiency in by-passing the PMF-dependent uptake without ROS contribution. Moreover, we show that silver allows aminoglycoside to kill highly recalcitrant anaerobic pathogen.

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