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2 **Silver potentiates aminoglycoside toxicity by enhancing their uptake**
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25

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27

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29 *E. coli*, *Clostridium difficile*

30 **Summary**

31

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

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48 Introduction

49
50 Increasing bacterial resistance to antibiotics is a major health issue, particularly in the
51 context of the low discovery rate for new antibiotics (Nordmann *et al.*, 2007). A solution to
52 this problem is to use antibiotic-potentiating or adjuvant agents to improve the efficacy of
53 existing antibiotics. Augmentin that combines a β -lactam and a β -lactamase inhibitor is a
54 well-known example of an antibiotic-adjuvant association, which has been used extensively to
55 treat infections caused by β -lactam resistant bacteria (Leigh *et al.*, 1981; Worthington and
56 Melander, 2013). Other types of potentiating effects can be obtained by facilitating the entry
57 of antibiotics into target bacterial cells (Mislin and Schalk, 2014).

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

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

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

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

95 **Results**

96

97 **Assessing the potentiating effects of metals on Gm toxicity**

98

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

122

123

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

125

126 We tested the potentiating activity of silver on an extended set of antibiotics from
127 diverse families. Silver was found to lower more than 10 fold the MIC values for all three
128 bactericidal aminoglycosides tested, i.e. kanamycin, tobramycin and streptomycin. A reduction
129 of only 2 fold was observed with spectinomycin, an aminoglycoside that is bacteriostatic (Fig.
130 1B). Similarly, a slight potentiating effect (less than 20% decrease of the MIC value) was

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

136
137

138 **Silver enhances Gm toxicity by acting independently of PMF**

139

140 Gm penetrates across the cytoplasmic membrane via a PMF-dependent process. We therefore
141 wondered whether PMF was required for the silver potentiating effect. To test this, we used
142 pharmacological and genetic approaches. Carbonyl cyanide-m-chlorophenylhydrazone
143 (CCCP), an uncoupler H⁺ ionophore, was used to dissipate the PMF. Then, we followed the
144 survival rate of *E. coli* after 1.5 hours of Gm treatment, in absence or in presence of CCCP
145 and with or without silver. As previously reported by us and others (Ezraty *et al.*, 2013;
146 Allison *et al.*, 2011), the presence of CCCP enhanced resistance of *E. coli* to Gm-mediated
147 killing, because it abolishes PMF. In contrast, adding silver reversed the effect and re-
148 sensitized *E. coli* even in the presence of CCCP (Fig. 2A). We also tested mutants known to
149 have low levels of PMF (Ezraty *et al.*, 2013). Specifically, we tested either a strain deleted of
150 *nuo* and *sdh* operons, which lacks complexes I and II, or a strain deleted of *iscUA* genes,
151 which poorly matures complexes I and II. Survival tests showed that $\Delta nuo \Delta sdh$ and $\Delta iscUA$
152 strains exhibited enhanced resistance to Gm (Fig. 2A). Consistently, MIC values of both
153 $\Delta nuo \Delta sdh$ (3.6 \pm 0.3) and $\Delta iscUA$ (6.1 \pm 0.5) strains were higher than wt (Fig. 2B). Adding
154 silver cancelled differences between mutant and wt strains. All three strains, wt, $\Delta nuo \Delta sdh$
155 and $\Delta iscUA$, showed the same survival loss of about 5 log in 1.5 hours (Fig. 2A) and all
156 three exhibited the same MIC value, i.e. 0.17 \pm 0.1 μ g/ml (Fig. 2B). Taken together, both
157 the pharmacological and genetic approaches demonstrated that silver enhanced Gm toxicity in
158 a PMF-independent manner.

159

160 **Silver restores Gm uptake in a PMF-deficient strain**

161

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

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

172

173 **Silver potentiating effect is EPD-II dependent**

174

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

181

182 **ROS production does not explain the positive combination between silver and** 183 **aminoglycosides**

184

185 Silver is known to destabilize Fe-S clusters (Liu and Imlay, 2013). Morones-Ramirez *et al.*
186 attributed potentiating effect of silver on aminoglycoside activity to enhanced production of
187 harmful ROS following Fe-S destabilization by silver, release of free iron and Fenton
188 chemistry (Morones-Ramirez *et al.*, 2013). To further investigate this issue, we tested the
189 silver-potentiating effect of Gm on anaerobically grown cultures. An *E. coli* strain grown in
190 anaerobic fermentative conditions exhibited enhanced resistance to Gm (Fig. 4A). However,
191 adding silver to anaerobically growing *E. coli* allowed Gm to cause a loss of 4 logs in
192 survival (Fig. 4A). This did not support the hypothesis of a contribution of ROS. We tested
193 the role of ROS in silver potentiating effect by using *E. coli* strains altered in their redox
194 homeostasis control. ROS-hyper-sensitive strains lacking superoxide dismutases (*ΔsodA*
195 *ΔsodB*) or the H₂O₂-stress responding master regulator (*ΔoxyR*) exhibited similar
196 sensitivity to silver potentiating effect as compared with the wild type parental strain (Fig.
197 4B, S2). Similarly, a strain expressing constitutively the H₂O₂-stress responding regulon
198 (*oxyRc*) exhibited a wild type-like sensitivity to the silver-potentiating effect of Gm (Fig.
199 4B, S2). Altogether, these observations indicate that ROS do not play a major role
200 in the Gm-potentiating activity of silver.

201

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

203 An *iscS* mutant exhibits enhanced resistance to silver (Fig S3A) (Morones-Ramirez *et al.*,
204 2013). A possibility put forward by others is that the *iscS* enhanced resistance was due to a
205 reduced level of Fe-S cluster biogenesis, hence a reduced possibility of fueling Fenton
206 chemistry upon Fe-S cluster destabilization (Morones-Ramirez *et al.*, 2013). However, *iscS*
207 mutation is pleiotropic and it is unsafe to interpret its phenotype as being the sole consequence
208 of an alteration of Fe-S cluster biogenesis. Therefore, we tested an *iscUA* mutant, which
209 is specifically altered in Fe-S cluster biogenesis. The *iscUA* mutant was hyper-sensitive to
210 silver (Fig. S3B). The reason for this is probably because silver targets Fe-S clusters in key
211 enzymes, whose decreased activities are detrimental for fitness, as this has already been
212 proposed (Xu and Imlay, 2012). In any case, the phenotype of *iscUA* mutant shows that
213 decrease in Fe-S cluster capacity sensitizes cells to silver toxicity, rather than protects it.
214 Then the question arose of why an *iscS* mutant still exhibits enhanced resistance to silver.
215 IscS degrades L-cysteine and L-cysteine is known like histidine to help *E. coli* to resist to
216 silver by sequestering it (Fig. S3C) (Matsumura *et al.*, 2003). Hence a possibility was that the
217 *iscS* mutant showed enhanced resistance to silver because it accumulated a higher
218 concentration of cysteine. To test this hypothesis, we over-produced CsdAE, a highly efficient
219 hetero-dimeric cysteine desulphurase, in an *iscS* mutant and observed that this did
220 suppress silver resistance of the *iscS* strain. As a control, we showed that a strain
221 synthesizing a mutated inactive version of CsdA was unable to act as a suppressor of the *iscS*
222 resistance (Fig. S3D). The data above suggest that Cys accumulation is the reason for the
223 hyper-resistance to silver of *iscS* mutant.

224

225 **Silver sensitizes *E. coli* Gm^R strain and aminoglycoside resistant** 226 **pathogens bacteria**

227

228 The *aacI* gene encodes a N-acetyltransferase that modifies and inactivates Gm. Accordingly,
229 an *E. coli aacI+* strain (Gm^R) exhibited a MIC value of 121 \pm 6 μ g/ml, i.e. ca. 50 fold higher
230 than wt and remained partially viable in the presence of 100 μ g/ml Gm (Fig. 5A and B).
231 Uptake experiments revealed that *E. coli aacI+* strain failed to accumulate intracellular Gm
232 (Fig. 5C). Remarkably, adding silver reduced the MIC value to 4.3 \pm 1, and provoked
233 bacteriostatic and bactericidal effects with 5 and 100 μ g/ml Gm treatment, respectively (Fig.
234 5A and B). Moreover, Gm uptake tests showed that silver allowed both Gm incorporation
235 and accumulation to resume in the Gm^R strain (Fig. 5C). Taken together, these results
236 showed that silver is able to sensitize a Gm^R strain to Gm treatment.

237 *Clostridium difficile*, an anaerobic enteropathogen causing infectious diarrhea, is well known
238 for its aminoglycoside resistance (Båverud *et al.*, 2004). Indeed, MIC values of *C. difficile* for
239 Gm, tobramycin and kanamycin were found to be very high (Fig. 5D). The question of the
240 silver potentiating capacity was therefore worth exploring. Spectacularly, in the presence of
241 silver, the MIC values of *C. difficile* dropped down from $43_{\pm 4}$ to $13_{\pm 3}$ $\mu\text{g/ml}$ for Gm, from
242 $115_{\pm 8}$ to $15_{\pm 4}$ $\mu\text{g/ml}$ for tobramycin and from $313_{\pm 26}$ to $13_{\pm 4}$ $\mu\text{g/ml}$ for kanamycin (Fig.
243 5D). Thus, overall treatment with silver decreased the MIC values for aminoglycosides from 3-
244 to 24-fold. Taken together, these results expanded the spectrum of silver potentiating effect to a
245 recalcitrant Gram-positive pathogen.

246 **Discussion**

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

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

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

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

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

306 Strict anaerobes are unable to efficiently take up aminoglycosides and therefore are resistant
307 to this family of antibiotics (Schlessinger, 1988) as observed for *C. difficile* (Båverud *et al.*,
308 2004). However, it is important to stress that ribosomes of clostridia can be bound and
309 inhibited by aminoglycosides (Bryan *et al.*, 1979). Our study points to a new approach to try
310 to fight vegetative cells of this bacterium, as silver was found to overcome the probable
311 natural lack of permeability of their membrane for aminoglycoside. This is an important
312 result as *C. difficile* infection is the most common cause of identifiable diarrhea in
313 hospitalized adult patients and the incidence and severity of *C. difficile* infections increased
314 (Lessa *et al.*, 2015 ; Rupnik *et al.*, 2009). In conclusion, our study depicts a new molecular
315 mechanism for the positive potentiating effect of silver on aminoglycoside activity, and shows

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

320

321 **Experimental procedures**

322

323 ***General microbiology and molecular biology***

324 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
 325 phosphate limiting minimal medium was the MOPS (pK_a 7.2) medium described by Neidhardt
 326 contained 0.1 mM K₂HPO₄ plus 9.8 mM KCl and 0.2 % glucose (Neidhardt *et al.*, 1974).
 327 P1*vir* transduction was used to move alleles into a different background. pCsdAE^{C358A} was
 328 constructed by site-directed mutagenesis using pCsdAE plasmid as template (Trotter *et al.*,
 329 2009), the oligonucleotides csdAE_C358A_F
 330 (CGGGCCGGGCAGCATGCCGCTCAGCCGCTAC) and csdAE_C358A_R
 331 (GTAGCGGCTGAGCGGCATGCTGCCCGGCCCG), *Pfu* Turbo DNA polymerase
 332 (Invitrogen) and *DpnI* from NEB. All plasmids were sequenced (Beckman Coulter Genomics)
 333 for verification.
 334

335

336 Strains	Relevant Genotype	Source or Reference
337 <i>E. coli</i> K-12 strains		
339		
340 MG1655	Parental strain	Lab collection
341 DV597	MG1655 Δ <i>iscUA</i> ::Cm ^r	(Vinella <i>et al.</i> , 2009)
342 LL111	MG1655 Δ <i>iscS</i> ::Cm ^r	(Trotter <i>et al.</i> , 2009)
343 BEFB20	MG1655 Δ <i>sdhB</i> Δ <i>nuo</i> :: <i>nptI</i> Kan ^r	(Ezraty <i>et al.</i> , 2013)
344 BEFB10	MG1655 <i>trp</i> ::Hfr::Gm ^r	(Ezraty <i>et al.</i> , 2013)
345 BEFB01	MG1655 Δ <i>sodA</i> ::Cm ^r Δ <i>sodB</i> ::Kan ^r	(Ezraty <i>et al.</i> , 2013)
346 BEFB02	MG1655 Δ <i>oxyR</i> ::Cm ^r	(Ezraty <i>et al.</i> , 2013)
347 QC2433	MG1655 <i>oxyRc</i>	Lab collection
348		
349 <i>C. difficile</i>		
350		
351 630 Δ <i>erm</i>		(Hussain <i>et al.</i> , 2005)
352		
353 Plasmids		
354		
355 pCsdAE		Lab collection (Trotter <i>et al.</i> , 2009)
356 pCsdAE ^{C358A}		This study

357 ***MIC determination***

358 The drugs tested were dissolved in LB medium for *E. coli* tests and in water for *C. difficile*
359 tests. The range of concentrations of each drug was twice the MIC. For *E. coli*, one hundred
360 microliters (100 μ L) of each 2X concentration tested were added in a 96-well microplate.
361 Each well was inoculated with 100 μ L of a fresh LB bacterial inoculum of 2×10^5 CFU/mL
362 and the plates were incubated at 37°C for 18 hours under aerobic conditions and agitation
363 150 rpm. *C. difficile* strain 630 Δ erm was grown anaerobically (5 % H₂, 5 % CO₂, and 90 %
364 N₂) in microplate. Each well was inoculated with 1 mL of a fresh BHI bacterial inoculum at
365 an OD₆₀₀ of 0.01 and the drug was added at different concentrations in the presence or
366 absence of AgNO₃ at 20 μ M. The plates were incubated at 37°C for 20 h under anaerobic
367 conditions. MIC was defined as the lowest drug concentration that exhibited complete
368 inhibition of microbial growth.

369

370 ***Lethality studies on LB agar plates***

371 Strains were grown aerobically (shaking 150 rpm) in minimal media M9 at 37°C. When the
372 OD₆₀₀ reached 0.2, serial dilutions of cell suspensions in phosphate buffer (0.05 M, pH 7.4) were
373 spread onto M9 media agar plates with different concentrations of silver nitrate and cysteine
374 (specified in each figures). C.f.u. were counted after incubation for 16 h at 37°C.

375

376 ***Metals***

377 For synergetic effect with antibiotics AgNO₃ was used at 8 μ M for *E. coli*, unless noted
378 otherwise, and 20 μ M for *C. difficile*, FeSO₄, CoCl₂, Cu₂(OAc)₄, Zn(OOCCH₃)₂ and NiCl₂
379 were used at 0.1 mM.

380

381 ***Survival rate***

382 Overnight cultures (16 h) were diluted 100 times and grown aerobically in LB at 37°C to an
383 OD₆₀₀ of 0.2. At this point, antibiotics and/or silver were added to the cells and after 1.5
384 h cells were taken, diluted in PBS buffer, spotted on LB agar and incubated at 37°C for
385 16 h. Cell survival was determined by counting colony-forming units per ml (c.f.u./ml). The
386 absolute c.f.u. at time-point 0 (used as the 100 %) was $\sim 5 \times 10^7$ cells/ml in all
387 experiments. When used, the PMF uncoupler, carbonyl cyanide-m-chlorophenylhydrazone
388 (CCCP) was added at a final concentration of 1 μ g/ml. When used, chloramphenicol (Cm)
389 was added 30 min before Gm treatment at a final concentration of 200 μ g/ml. The Cm stock
390 solution was extemporaneously prepared at 20 mg/ml with 1/3 ethanol and 2/3 H₂O.
391 Synergic effect in anaerobic conditions was performed as previously described but in

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

394 For silver sensitivity experiments M9 minimal media was used instead LB in order to
395 minimized the variability of the results due to the chelation of the metal by medium
396 components present in the LB (Feng *et al.*, 2000). Overnight cultures (16 h) were diluted 100
397 times and grown aerobically in minimal media M9-0.2 % glycerol at 37°C to an OD₆₀₀ of
398 0.2. At this point, AgNO₃ was added to the cells and after different incubation times (specified
399 on each figure) cells were taken, diluted in phosphate buffer (0.05 M, pH 7.4), spotted on LB
400 agar and incubated at 37°C for 16 h.

401

402 ***Gm uptake***

403 [³H]-Gm (20 µCi/mg; Hartmann Analytic Corp.) was added to a final concentration of
404 5 or 0.25 µg/ml and cultures were incubated at 37°C on a rotary shaker. At given times,
405 500 µl aliquots were removed and collected on a 0.45 µm-pore-size HAWP membrane filter
406 (Millipore) pretreated with 1 ml of unlabeled Gm (250 µg/ml). Filters were subsequently
407 washed with 10 ml of 3 % NaCl, placed into counting vials, dried for 30 min at
408 52°C whereafter 8 ml of scintillation liquid were added and incubated overnight at room
409 temperature. Vials were counted for 5 min. Gm uptake efficiency is expressed as total
410 accumulation of Gm (nanograms) per 10⁸ cells.

411

412 ***Statistical analysis***

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

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506

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518

519 **Legends:**

520

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

522

523 (A) Minimal Inhibitory Concentration (MIC) of Gm (in $\mu\text{g/ml}$) in the presence of different
524 metals was calculated. *E. coli* strain MG1655 was grown in LB under aerobic conditions in
525 presence of 8 μM AgNO_3 , or 0.1 mM FeSO_4 , CoCl_2 , $\text{Cu}_2(\text{OAc})_4$, $\text{Zn}(\text{OOCCH}_3)_2$ and NiCl_2 .
526 MIC values were calculated as described in Materials and Methods. (B) MIC values of *E. coli*
527 MG1655 for different antibiotics as indicated on top of the graphs. AgNO_3 concentration used
528 was 8 μM . * $P \leq 0.05$; ** $P \leq 0.01$; and *** $P \leq 0.001$ (Mann-Whitney U test).

529

530

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

532 (A) Percentage survival of *E. coli* MG1655 after 1.5 h treatment with Gm (5 $\mu\text{g/ml}$) and/or
533 AgNO_3 (8 μM). Genotypes of strains studied (wt, $\Delta nuo \Delta sdh$ and $\Delta iscUA$) are indicated on
534 top of the graph. Wt strain was also studied after growth in the presence of CCCP (1
535 $\mu\text{g/ml}$) as indicated. Black, dark grey, light grey and red bars are for untreated, AgNO_3 , Gm
536 and Gm and AgNO_3 together. (B) MIC values of wt, $\Delta iscUA$ and $\Delta nuo \Delta sdh$ strains grown in
537 the presence of Gm (dark bar) or Gm and AgNO_3 (8 μM) (red bar). (C) Presence of silver
538 restore tritiated Gm ($^3\text{H-Gm}$) uptake in $\Delta iscUA$ and $\Delta nuo \Delta sdh$ mutants. Uptake was
539 measured by incubating early exponential-phase cultures ($\text{OD}_{600} \sim 0.2$ with 5 $\mu\text{g/ml}$ $^3\text{H-Gm}$ in
540 presence or absence of AgNO_3 (8 μM) at 37°C. (D) Gm uptake test with sub-lethal Gm
541 concentration. The wt strain was grown up to $\text{OD}_{600} \sim 0.2$ with 0.25 $\mu\text{g/ml}$ $^3\text{H-Gm}$ in the
542 presence or absence of AgNO_3 (8 μM) at 37°C. * $P \leq 0.05$; ** $P \leq 0.01$; and *** $P \leq 0.001$
543 (Mann-Whitney U test).

544

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

547 Percentage survival of *E. coli* MG1655 after 1.5 h treatment with Gm (5 $\mu\text{g/ml}$) and/or
548 AgNO_3 (8 μM) with or without chloramphenicol (Cm). Strain was grown in the presence of
549 saturating concentration of Cm (200 $\mu\text{g/ml}$) for 30 min before adding Gm and/or AgNO_3 .
550 Black, dark grey, light grey and red bars represent untreated, AgNO_3 -treated, Gm-treated and
551 Gm + AgNO_3 -treated cultures.

552

553

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

556 (A) Percentage survival of an *E. coli* MG1655 after 1.5 h treatment with AgNO₃ (dark grey
557 bar) Gm (light grey bar), Gm + AgNO₃ (red bar) in anaerobic condition. Gm was used at 5
558 µg/ml and silver at 8 µM. Anaerobic condition was realized in anaerobic chamber (Coy
559 Chamber; 5 % H₂, and 90 % N₂) with anaerobic LB media previously equilibrated in the
560 anaerobic chamber for 24 h. (B) Percentage survival of *E. coli* MG1655 after 1.5 h
561 treatment with AgNO₃ (dark grey bar) Gm (5 µg/ml) (light grey bar), Gm + AgNO₃ (2 µM)
562 (blue bar), Gm + AgNO₃ (4 µM) (yellow bar), Gm + AgNO₃ (6 µM) (green bar), Gm +
563 AgNO₃ (8 µM) (red bar) in aerobic condition. Genotypes of strains studied (wt, $\Delta sodA$
564 $\Delta sodB$, $\Delta oxyR$ and $oxyR^C$) are indicated on top of the graphs. *P ≤ 0.05 and **P ≤ 0.01
565 (Mann-Whitney U test).

566

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

568

569 (A) MIC values (µg/ml) of an *E. coli* Gm^R mutant (*aacI*+ strain) grown in presence of
570 AgNO₃ (8 µM). (B) Percentage survival of an *E. coli* Gm^R mutant after 1.5 h treatment with
571 AgNO₃ (dark grey bar) Gm (light grey bar), or Gm and silver together (red bar). Gm was
572 used at 5 or 100 µg/ml, as indicated, and AgNO₃ 8 µM. (C) Gm uptake test. The Gm^R mutant
573 was grown up to OD₆₀₀ ~ 0.2 with 5 µg/ml ³H-Gm in the presence or absence of AgNO₃ (8
574 µM) at 37°C. (D) MIC in µg/ml of *C. difficile* strain 630 Δerm was calculated for different
575 antibiotics. Strain 630 Δerm inoculated at an OD₆₀₀ of 0.01 was grown for 20 hours in the
576 presence or absence of 20 µM AgNO₃ and in the presence of increasing concentrations of Gm,
577 tobramycin and kanamycin. After 20 h of incubation at 37°C, the OD₆₀₀ of the strain
578 630 Δerm grown in the absence of antibiotics was similar in the presence or absence of 20
579 µM. *P ≤ 0.05; **P ≤ 0.01; and ***P ≤ 0.001 (Mann-Whitney U test).

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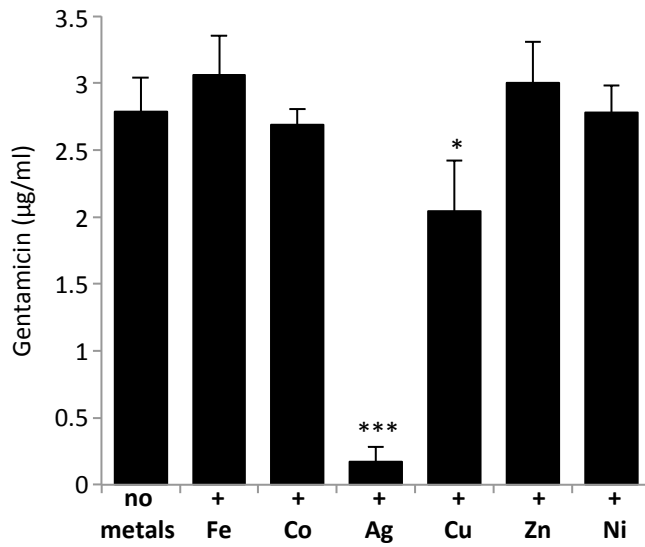
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Fig. 1

A



B

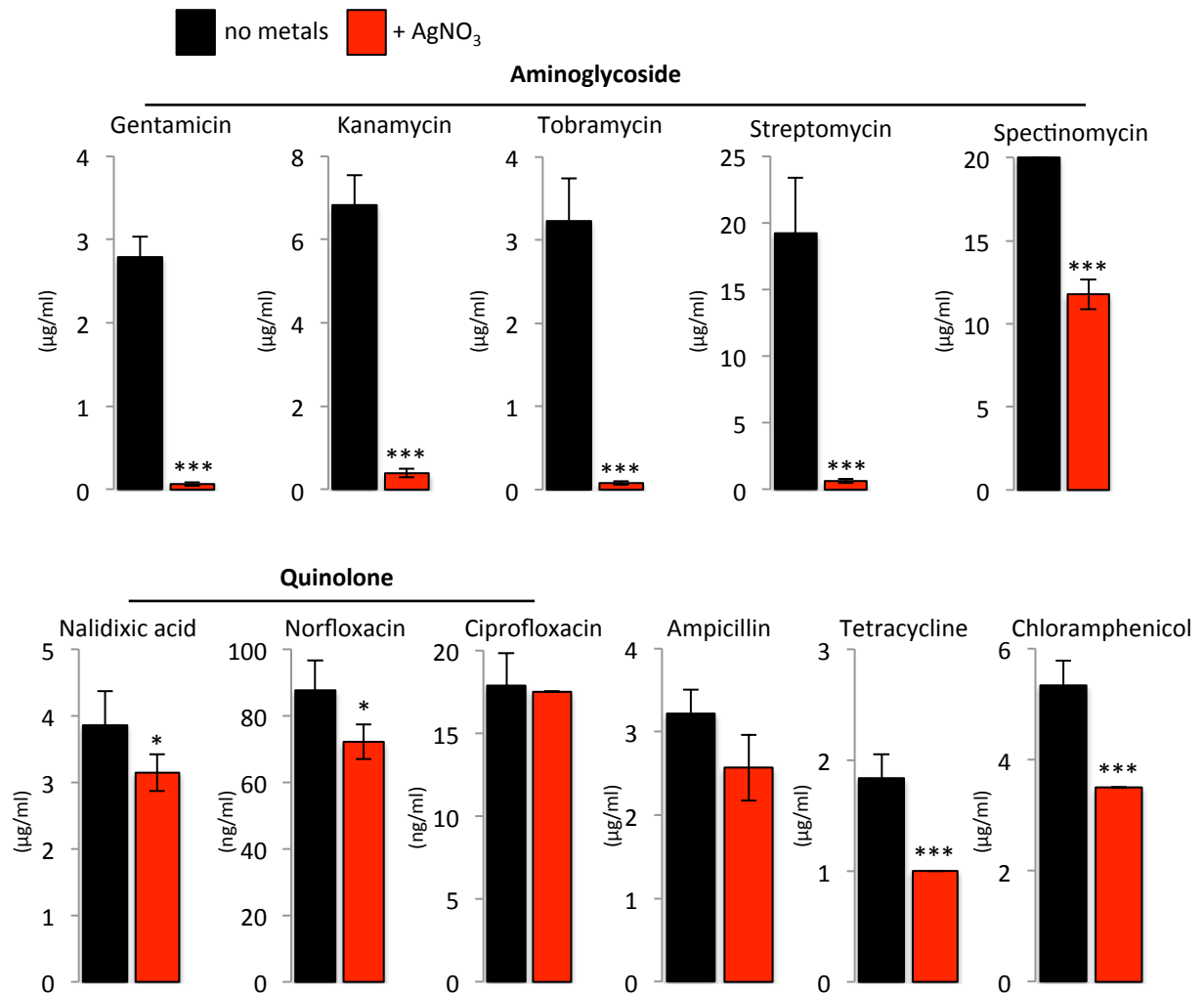


Fig. 2

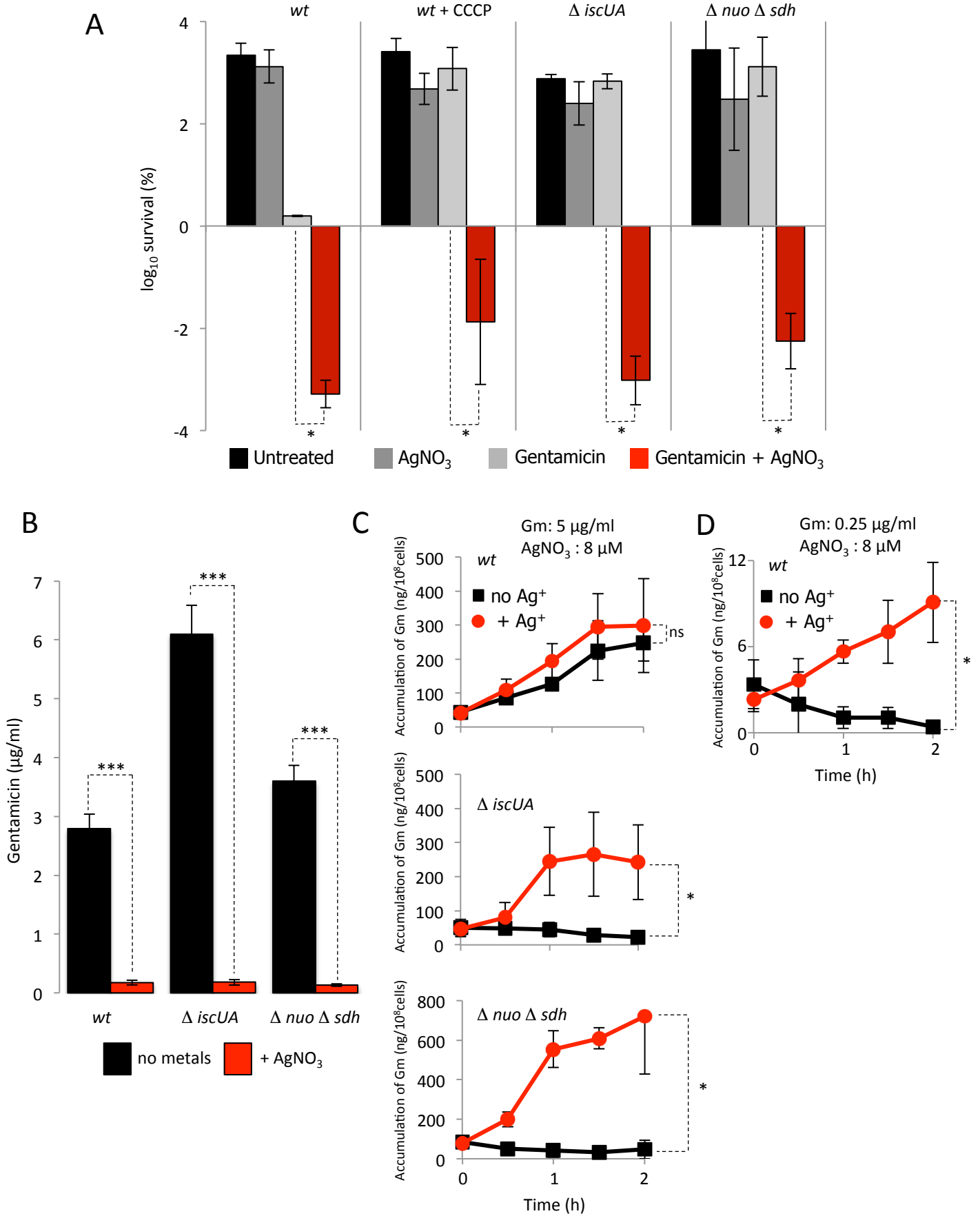


Fig. 3

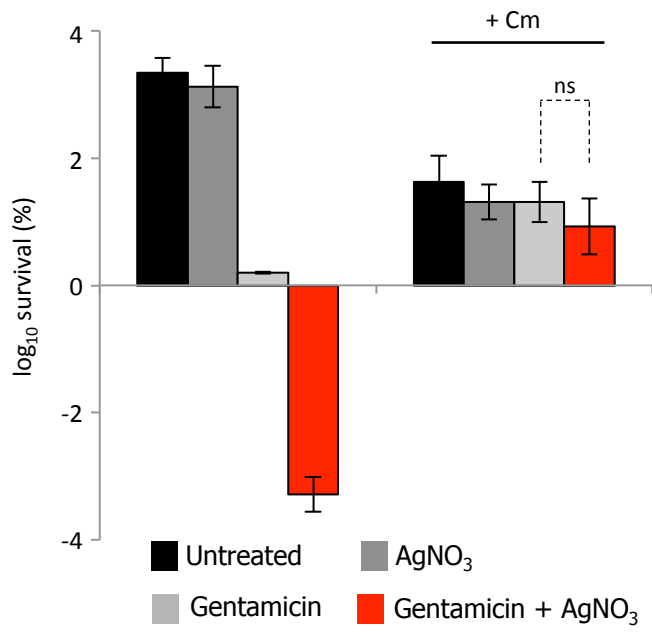


Fig. 4

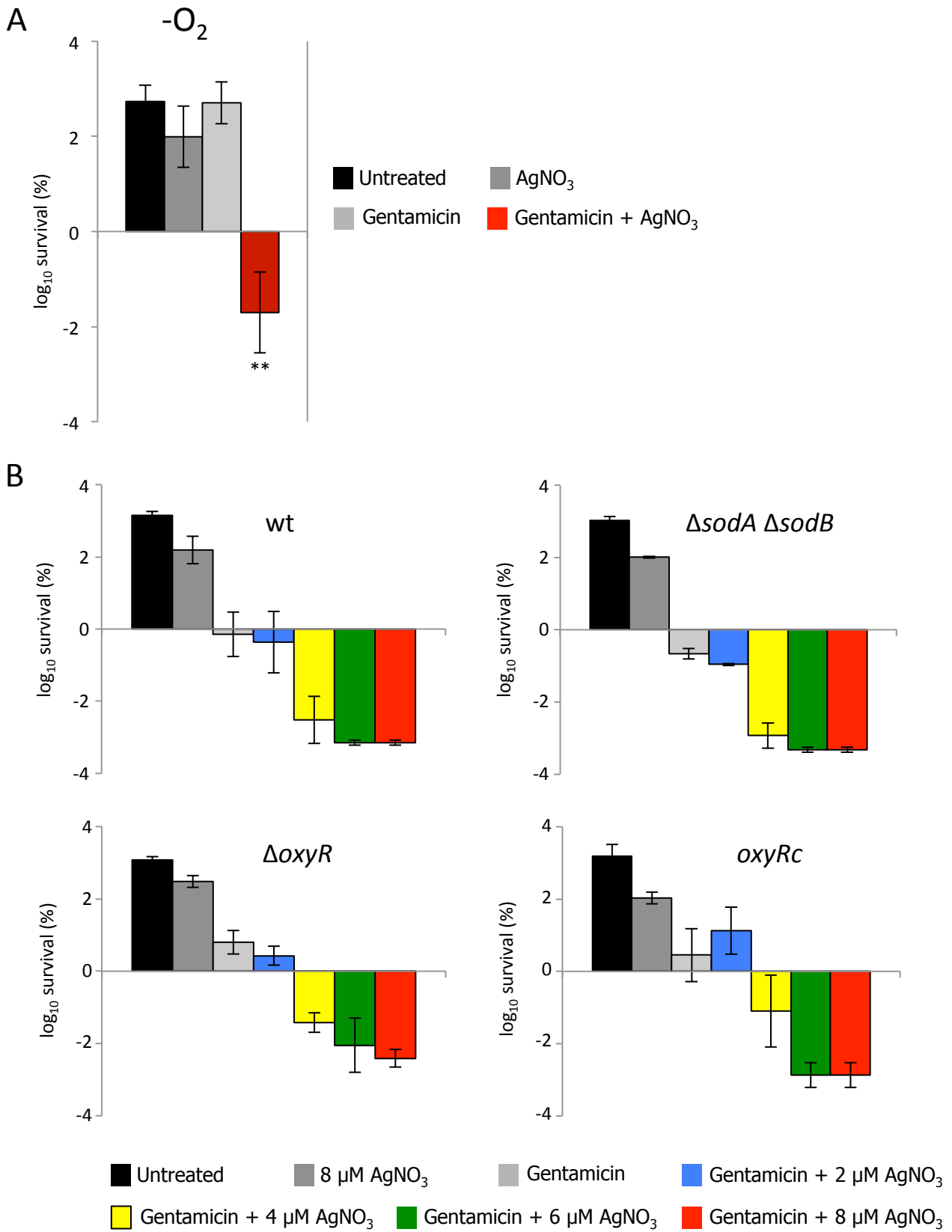
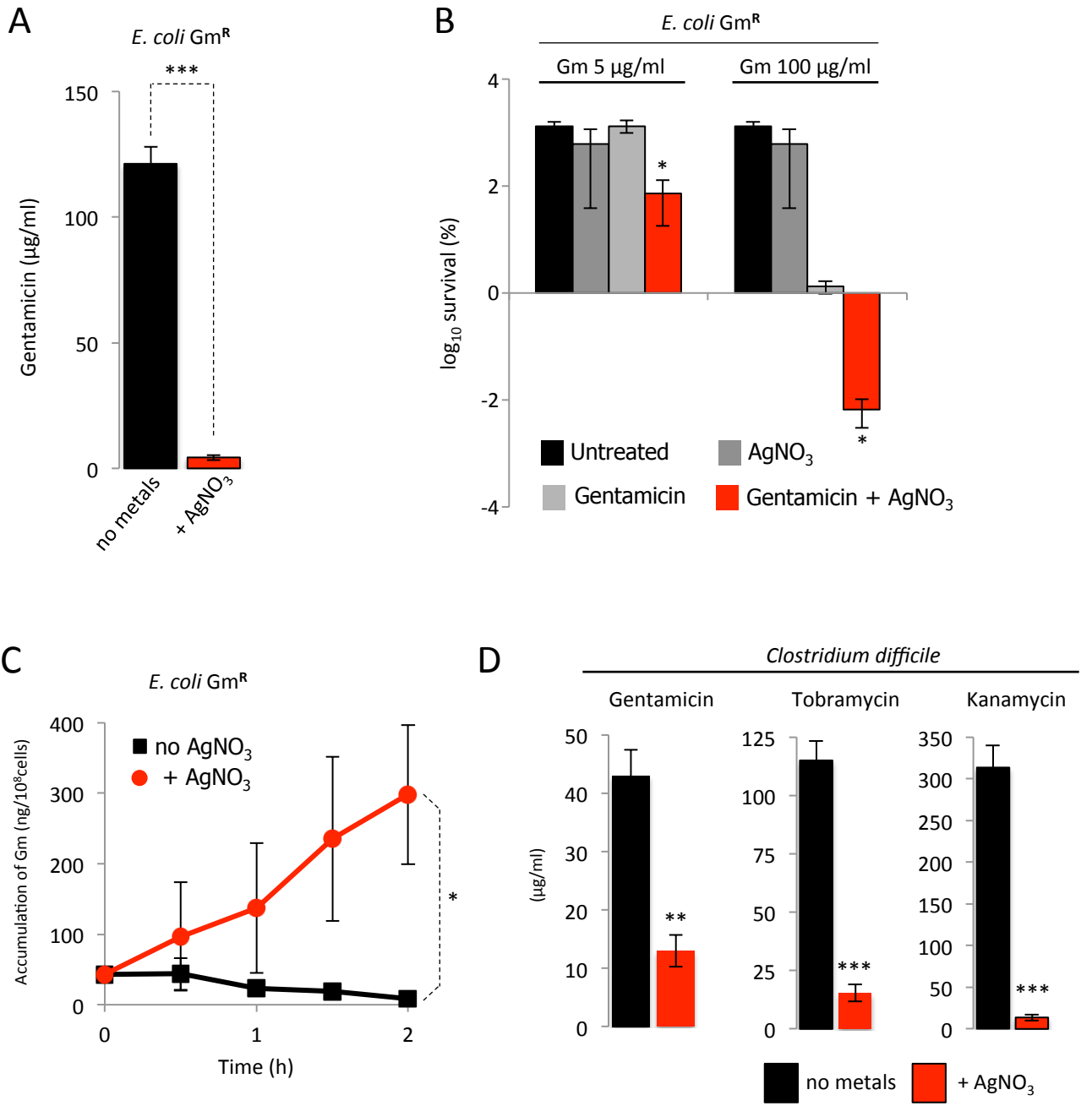


Fig. 5



Silver potentiates aminoglycoside toxicity by enhancing their uptake

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Supplementary Information

Figure Legends

Figure S1. The medium influences the silver potentiating effect

(A) MIC values ($\mu\text{g/ml}$) of Gm for an *E. coli* wt strain grown in LB under aerobic conditions in presence of different concentrations of AgNO_3 . Black, blue, yellow, green and red bars represent conditions with no metals, 2, 4, 6 and 8 μM AgNO_3 , respectively. (B) MIC values of Gm ($\mu\text{g/ml}$) for an *E. coli* wt strain growing in M9 minimal medium in presence of AgNO_3 (4 nM) or $\text{Cu}_2(\text{OAc})_4$ (0.25 μM). (C) MIC values of Gm ($\mu\text{g/ml}$) for an *E. coli* wt strain growing in M9 minimal medium supplemented with 0.2% Casamino acids in presence of AgNO_3 (50 or 100 nM). (D) Percentage survival of *E. coli* MG1655 grown in M9 minimal medium supplemented with 0.2% Casamino acids after 1.5 h treatment with Gm (2, 3 and 5 $\mu\text{g/ml}$) and/or AgNO_3 (50 and 250 nM). Black, yellow and red bars represent cultures untreated, AgNO_3 (50 nM) treated and AgNO_3 (250 nM) treated, respectively. Gm concentrations are indicated on bottom of the graph. (E) MIC values of Gm ($\mu\text{g/ml}$) for an *E. coli* wt strain growing in Neidhardt's low Pi medium in presence of AgNO_3 (10 nM). (F) Percentage survival of *E. coli* MG1655 grown in Neidhardt's low Pi medium after 1.5 h treatment with Gm (0.3 $\mu\text{g/ml}$) and/or AgNO_3 (10 nM). Black, dark grey, light grey and red bars represent cultures untreated, AgNO_3 treated, Gm treated and Gm + AgNO_3 treated, respectively. * $P \leq 0.05$; ** $P \leq 0.01$; and *** $P \leq 0.001$ (Mann-Whitney U test).

Figure S2. Silver potentiating effect is independent of the level of ROS production.

MIC values of Gm ($\mu\text{g/ml}$) for *E. coli* wt and anti-ROS mutant strains grown in LB under aerobic conditions in presence of AgNO_3 (8 μM). Genotypes of strains studied (wt, ΔsodA ΔsodB and oxyR^{C}) are indicated on top of the graph.

Figure S3. Fe/S clusters destabilization does not explain the positive combination between silver and aminoglycoside

(A) Survival of *E. coli* MG1655 and ΔiscS exposed to AgNO_3 (13 μM). Genotypes of strains studied (wt and ΔiscS) are indicated on the graph. Black and red lines are for untreated and AgNO_3 -treated cells. (B) The *iscUA* mutant is more sensitive to silver.

Dilutions of wild type and *iscUA* strains cultures were spotted on M9 medium added with, or not, silver as indicated above the figure. Growth was recorded after 20 h incubation at 37°C. (C) Cysteine protects *E. coli* against silver stress. Dilutions of wild type strain culture were spotted on M9 medium added with increasing concentrations of silver and cysteine as indicated on the figure. Growth was recorded after 20 h incubation at 37°C. (D) Percentage survival of $\Delta iscS$ mutant carrying an empty vector (pBad24) or a vector containing *csdE* gene (encoding a hetero-dimeric cysteine desulfurase) and *csdA* gene (wild type or mutated allele) after 2 h treatment with AgNO₃ (2 μM) in M9 media.

Table S1. MIC values for MG1655 *E. coli* strain

In LB medium

Metals	Products	Minimal inhibitory concentration (MIC)	Concentrations used in Fig. 1A
Iron (Fe)	FeSO ₄	> 5 mM	0.1 mM
Cobalt (Co)	CoCl ₂	2.5 mM	0.1 mM
Silver (Ag)	AgNO ₃	70 μM	8 μM
Copper (Cu)	Cu(CO ₂ CH ₃) ₂	> 5 mM	0.1 mM
Zinc (Zn)	Zn(CO ₂ CH ₃) ₂	1.5 mM	0.1 mM
Nickel (Ni)	NiCl ₂	2.5 mM	0.1 mM

In M9 minimal medium

Metals	Products	Minimal inhibitory concentration (MIC)	Concentrations used in Fig. S1
Silver (Ag)	AgNO ₃	40 nM	4 nM
Copper (Cu)	Cu(CO ₂ CH ₃) ₂	3 μM	0.25 μM

In M9 minimal medium + CASA (0.2 %)

Metals	Products	Minimal inhibitory concentration (MIC)	Concentrations used in Fig. S1
Silver (Ag)	AgNO ₃	500 nM	50; 100; 250 nM

In low phosphate minimal medium

Metals	Products	Minimal inhibitory concentration (MIC)	Concentrations used in Fig. S1
Silver (Ag)	AgNO ₃	0.1 μM	10 nM

Fig. S1

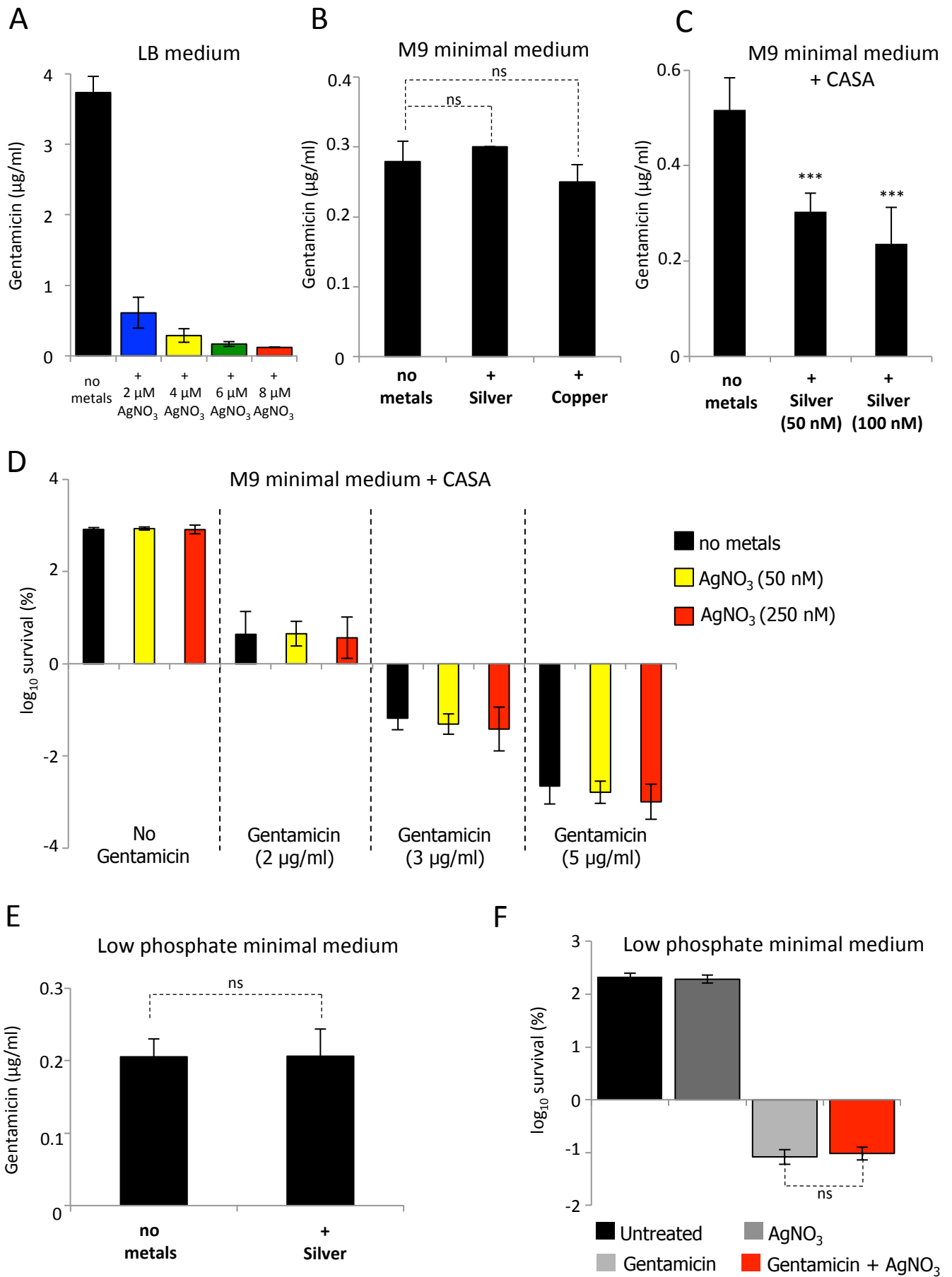


Fig. S2

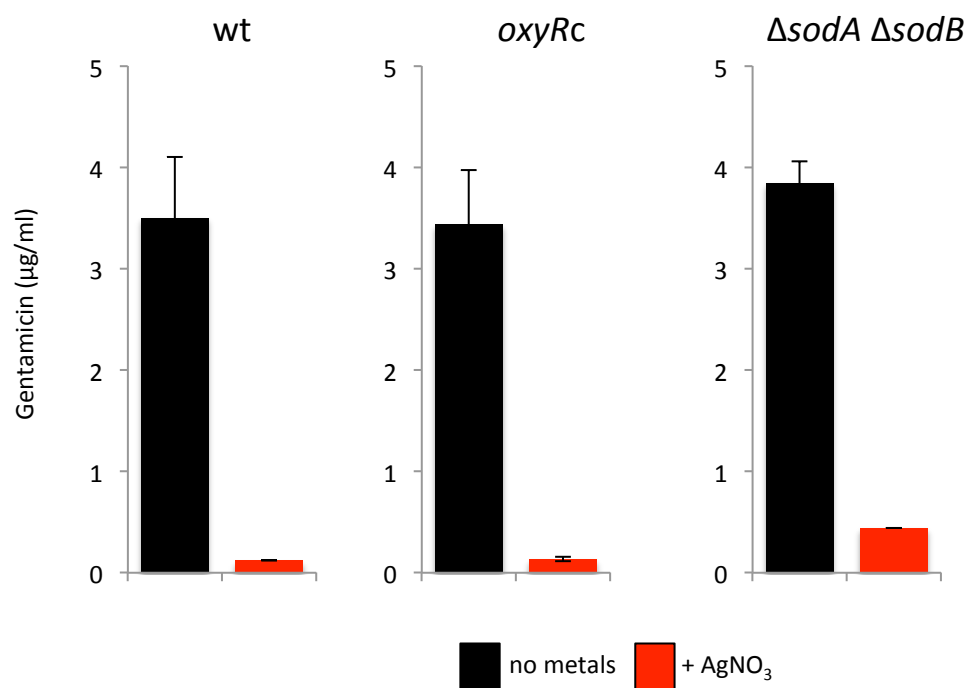


Fig. S3

