Experimental evolution of *Escherichia coli* on solid silver, copper, stainless steel, and glass surfaces

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Abstract

To study bacterial adaptation to antimicrobial metal surfaces in application-relevant semi-dry environmental conditions, *Escherichia coli* ATCC 8739 was exposed to copper, silver, stainless steel, and glass surfaces for thirty consecutive cycles in exposure conditions designed to select for either survival or growth. Subsequent mutation analysis did not find changes in overall mutation frequency that could lead to enhanced adaptation potential nor mutations in *cop, cus, cue, sil, pco* or general efflux genes known to actively maintain copper/silver homeostasis in copper or silver exposed populations compared to populations evolved on steel and glass control surfaces. However, mutations in *silS* rapidly activated the cryptic *sil* efflux locus during silver ion challenge in liquid assay with the evolved populations and caused collateral increase in ampicillin and ciprofloxacin tolerance, indicating that efflux might be specific to granting heavy metal tolerance in liquid but not surface exposure format. Instead, during cyclic surface exposure mutations in genes related to cellular barrier functions and sulfur metabolism were enriched on copper and silver surfaces. This increased metal surface tolerance but had less to no protective effect in subsequent conventional MBC and MIC tests indicating that reducing bioavailability and passively restricting uptake of the toxic metals rather than active efflux contributes to increased viability on copper and silver surfaces compared to liquid format. Our findings highlight the critical importance of appropriate exposure conditions not only in efficacy assessment but also risk assessment of antimicrobial surfaces rendering results acquired via liquid tests not applicable for dry-use surfaces.

Importance

This study examines the evolutionary adaptations of *Escherichia coli* under sublethal exposure to copper and silver surfaces, revealing a moderate increase in surface tolerance but no heightened mutation frequency or enhanced metal ion tolerance in standard tests. Notably, enriched mutations indicate a shift toward more energy-passive mechanisms of metal tolerance. Additionally, while enhanced silver efflux—with a collateral increase in antibiotic tolerance—was rapidly selected in a single round of silver exposure in liquid tests and substantially increased copper and silver ion tolerance in conventional test formats, the causal mutations did not improve viability on silver and copper surfaces, underscoring the different fitness dynamics of tolerance mechanisms dependent on exposure conditions. These findings emphasize the need for appropriate exposure conditions in evaluating of both efficacy and the potential risks of using antimicrobial surfaces, as the results from conventional liquid-based tests may not apply in solid contexts.

Introduction

Antimicrobial surfaces are expected to passively reduce bioburden on surfaces with elevated risk of pathogen transmission. Among the materials used, silver and copper based applications have the longest application history as well as wide commercial use [1]. Despite challenges in efficacy assessment

of such materials in environmentally exposed conditions there is a wide range of environmentally directed efficacy scenarios with highly variable proportions of microbial survivors from quick killing of most microbes to effectively no biocidal activity [2]. That raises a concern that prolonged or repeated sublethal exposure to antimicrobial surfaces could not only increase biocide tolerance but also cause antibiotic resistance in these microbes.

Increased risk of antibiotic resistance could arise from co-selection, cross-selection or co-regulation of molecular mechanisms that increase both antimicrobial metal as well as antibiotic tolerance. The risk of co-selection of metal tolerance and antibiotic resistance is obvious and inevitable in the case of large multiresistance plasmids [3], [4] but in case of cross-resistance and co-regulation, test conditions might substantially affect selection. Although cross-selection of mutations in efflux systems and outer membrane proteins has been well described in decreased sensitivity to metals and antibiotics (*e.g.* [5], [6], [7]) the risk of *de novo* resistance development, potential cross-selection of beneficial traits in multistress exposure, and effect of non-genetic co-regulation are more challenging to study.

Mutation rate of an evolving population can naturally fluctuate in time due to the fixation of various hypermutator and antimutator alleles [8], [9], [10] as also demonstrated in the Lenski long-term evolution experiment (LTEE) with *Escherichia coli* [11]. At the same time, presence of the hypermutator phenotype has been associated with increased pathogenicity [12], [13], [14], [15], [16], [17] and antibiotic resistance development [18], [19], [20], [21]. As shown previously, in growth-supporting exposure media subinhibitory copper and silver could increase mutation rate [22], [23] and heavy metals could thereby lead to antibiotic resistance development [24]. However, the role of metal-based biocides in *de novo* antibiotic resistance development or their effect on mutation rate is controversial, especially in lethal and/or low-organic conditions. Similarly to antimicrobial surface efficacy, molecular defense mechanisms potentially leading to cross-resistance or coregulation are possibly highly dependent on biotic and abiotic environment as well as the resources available to the bacteria. While it has been feared that antimicrobial agents that increase mutation frequency could collaterally enhance antibiotic resistance development and increase pathogenicity there are also examples where the contrary is demonstrated and some metal ions such as copper and zinc can suppress not only hypermutation but also conjugative gene transfer possibly by blocking the SOS response [25], [26], [27]. In addition, one must keep in mind that majority of the published data is acquired from homogenous liquid environment which do not necessarily resemble surface properties other than release of the active ingredient. Instead, the antimicrobial effect on *e.g.*, copper-based surfaces is rather dependent on physical properties of the surface material such as its copper content of the alloy and direct surface-microbe contact not the amount copper released from the surface[28]. It has also been shown that the speed of contact-killing on copper surfaces is linked to bacterial cell surface components and their interactions with the metal surface indicating limited ability to evolve resistance to metallic copper [29]. On air-exposed solid surfaces, bacteria are not only exposed to the biocide incorporated into the surface material but also several other environmental stressors such as desiccation, light exposure, osmotic shock, starvation etc. The specific stress responses mechanistically overlap with response to metal ions (Fig 1) highlighting possible co-regulational toll or benefit associated with combined exposure. In contrast to subinhibitory copper concentrations in liquid growth medium, transient exposure to copper surfaces in environmentally exposed non-growth supporting conditions has been shown not to increase mutation frequency of *E. coli* [30].

Evolution of antimicrobial tolerance on solid surfaces is a rather novel topic with two articles recently published on the matter. Those studies by Bleichert *et al.* [31] and Xu *et al.* [32], investigated evolution only on copper surfaces using different approaches to controls, exposure and growth conditions. With the current study we aimed to illustrate the importance of environmental conditions on different evolutionary scenarios in bacteriostatic and bactericidal conditions, more specifically to adaptation on copper and silver surfaces in either high-organic humid (HH) or low-organic dry (LD) conditions. The use of *Escherichia coli* strain ATCC 8739 harboring a pathogenicity-associated [33] cryptic chromosomal copper and silver tolerance island [34], [35] with mutational activation [35], [36] in laboratory evolution experiments was expected to offer a good model for additionally assessing the role of efflux in metal detoxification.

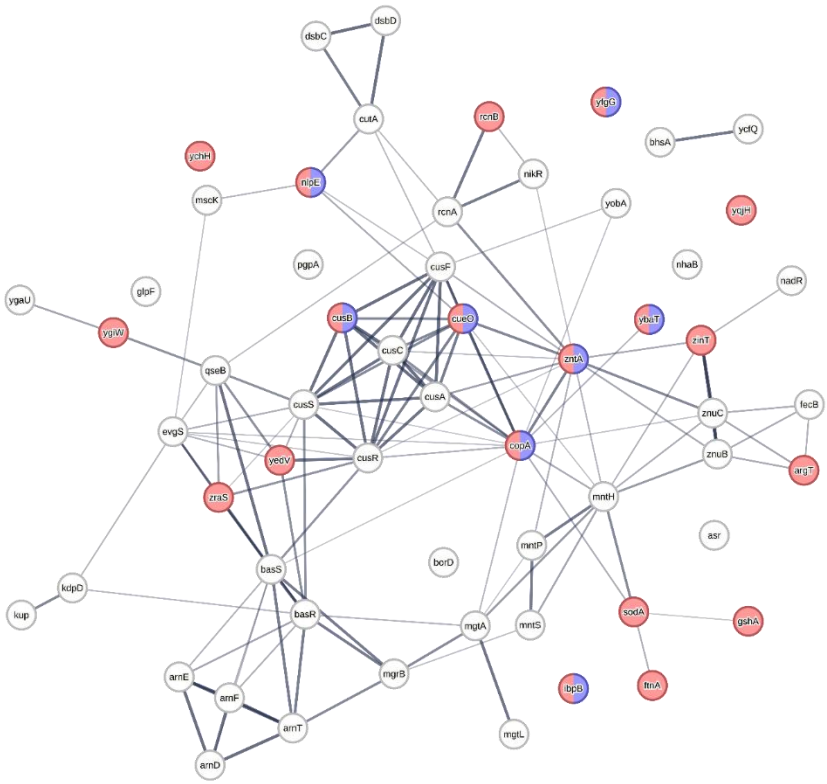

Figure 1. Known and predicted interactions of the *E. coli* genes with relevant Gene Onthology (GO) functional annotations [37] retrieved from EMBL STRING [38], default settings. Interactions of the genes belonging to the GO term “response to metal ion” (GO:0010038). Overlapping genes with the GO term “response to stress” (GO:0006950) and “stress response to metal ion (GO:0097501) are marked in red and blue, respectively. Line thickness indicates the strength of data support for the interaction.

Materials and Methods

Strains and media

Escherichia coli strain routinely used for efficacy testing of antibacterial agents was used in all experiments. *E. coli* DSM 1576 (ATCC 8739, NCIB 8545, WDCM 00012, Crooks strain) was freshly ordered from the DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). 2nd subculture of the received strain on LB agar medium (Table 1) was used for the evolution experiments. Here onwards, the more widely used synonymous ATCC 8739 strain number is used.

Surface treatment

99% copper (Metroprint OY, Estonia) and 99.95% silver (Surepure Chemetals, USA) were used as historically well-known antimicrobial materials. Stainless steel (AISI 304 SS, incl. 18% Cr, 8% Ni, 1.4% Mn; 2B finish; Aperam-Stainless, France) is a widely used metallic surface and has been frequently suggested as a control surface in studies involving metal alloys as well as standard biocide testing methods. 18x18 mm borosilicate glass microscopy cover slips were used as a biologically inert control surface (Corning Inc., USA). Steel, copper, and silver metal sheets were laser-cut to Ø 2 cm coupons and reused in evolution experiments. After each exposure the metal coupons were collected by type and inactivated in 70% ethanol for 15 minutes. The cleaning cycle consisted of 3 sequential solution steps (5% citric acid, acetone, 70% EtOH) with glass beads and 2 rinsing steps without glass beads. Solutions steps included ultrasonication in water bath for 15 minutes followed by shaking for 2 minutes manually before and after ultrasonication. Between each solution step the coupons were rinsed in distilled water by shaking. After cleaning the coupons were dried in a biosafety cabinet and stored in sterile Petri dishes. Glass surfaces were rinsed with 70% EtOH, dried and exposed to UV-C radiation for 15 min on both sides.

Evolution experiments

The two evolution experiments (Table 1) each consisted of three parts: (re-)inoculation, exposure in the climate chamber and harvesting of cells for plating and reinoculation repeated in 30 cycles. In each cycle, a sample from each inoculum was preserved in glycerol stock at -80°C to prevent loss of lineages due to contamination and/or lethal exposure. After the 30th cycle the collected colony suspension was also stored at –20° C for DNA extraction.

For cyclic exposure sterile test surfaces were placed into the wells of a 6-well plate. In case of humid exposure, a sterile 3D printed plastic grid was used to suspend each test surface above 3 ml of sterile water pipetted below the suspension grid to preserve moisture in the system.
For the first cycle of each experiment *E. coli* ATCC 8739 was inoculated on LB agar plates from glycerol stock culture and incubated for 24 h at 37° C from which a single colony was further inoculated on LB agar for 20 h 37° C. From the resulting second subculture all colonies were collected by suspending with 5 ml of phosphate buffered saline (PBS). 500 µl of that suspension was preserved in glycerol stock and sequenced as the “ancestor”. For 2nd to 30th exposure cycle, inoculum was made from resuspended colonies from the previous cycle plate-out. Prior to inoculation, the cell density was spectrophotometrically adjusted to 3.3×10^8 CFU/ml or 1.6×10^9 CFU/ml [39] for exposure with EPA soil load (SL, [40]) or 500-fold diluted nutrient broth (NB, [41]), respectively (Table 1). The inoculum was diluted 1:1 with 2x SL or with 250-fold diluted NB resulting in final target cell count of 8.1×10^6 CFU/surface in both evolution experiments. Surfaces were inoculated with 50 µl droplet of inoculum in

157 case of SL and 5x2 µl droplets of inoculum in case of diluted NB (Table 1). Inoculated surfaces were
158 transferred into a climate chamber (Climacell EVO, Memmert, USA)) and exposed for a defined time at
159 defined climatic conditions (Table 1). Exposure was stopped by submerging each test surface to a 50 ml
160 centrifuge tube containing 10 ml soy casein digest lecithin polysorbate broth (SCDLP, [41]) and vortexing
161 for 30 seconds at full speed. 500 µl of the harvested suspension and its 10^{-1} and 10^{-2} dilutions were
162 bead-plated on LB agar and incubated for 40-50 h at 37°C. Lowest dilution with non-confluent colony
163 growth was used to prepare the next inoculum.
164
165 Mutants carrying mutations of interest were isolated from evolved populations and colonies that
166 tolerated substantially higher silver salt concentration in the minimal biocidal concentration (MBC)
167 assays after the evolution experiment. Those isolates were subjected to whole genome sequencing as
168 described below and included in the metal and antibiotic tolerance testing.
169
170 **Table 1**. Experimental conditions used in the evolution experiments.

	Experiment 1: **High-organic humid (HH) conditions**				Experiment 2: **Low-organic dry (LD) conditions**			
Organism	*Escherichia coli* ATCC 8739							
Inoculum cell count	8.1×10^6 CFU per surface							
Exposure temperature	22°C							
Exposure medium	Organic proportion of EPA soil load [40](6.8 g/l organics): bovine serum albumin 2.5 g/l yeast extract 3.5 g/l mucin 0.8 g/l				500-fold diluted nutrient broth [41] (0.026 g/l organics): 0.006 g/l meat extract 0.02 g/l peptone 0.01 g/l NaCl			
Inoculum volume	1 x 50 µl				5 x 2 µl			
Relative humidity	90%				50%			
Surface material	Copper	Silver	Steel	Glass	Copper	Silver	Steel	Glass
Exposure duration	1 h	6 h	6 h	6 h	20-25 min	2 h	2 h	2 h
Toxicity-neutralizing harvesting medium	Soy casein digest lecithin polysorbate broth (SCDLP [41]): 17 g/l casein peptone, 3 g/l soybean peptone, 5 g/l NaCl, 2.5 g/l Na_2HPO_4, 2.5 g/l glucose, 1.0 g/l lecithin, 7.0 g/l Tween80							
Medium for clonal growth between exposures	LB agar medium: 5 g/l yeast extract, 10 g/l tryptone, 5 g/l NaCl, 15 g/l agar							
Time of clonal growth between exposures	~40 h				~45 h			
Harvesting medium for populations from clonal growth plates for reinoculation	Phosphate-buffered saline (PBS): 10-fold diluted stock of: 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4; pH 7.1							

171

172 **Copper, silver and antibiotic tolerance**

173 In the test formats described below bacterial endurance of silver, copper or antibiotic challenge was
174 assessed in the context of which the terms resistance and tolerance need to be clarified. Antibiotic
175 resistance is well defined by change of minimal inhibitory concentration (MIC) exceeding epidemiologic
176 cut-off values for general microbiology or clinical breakpoints for susceptibility testing in clinical context
177 [42], [43]. However, use of the term in the context of other antimicrobials, including antimicrobial
178 metals, is often confusing. For biocidal antimicrobials, killing of the microbes measured by minimal
179 biocidal concentration (MBC) is a more relevant parameter than MIC. By this definition, metal resistance
180 is rare, possibly due to multiple mechanisms of action as opposed to limited mode of action of most
181 antibiotics more easily counteracted by specific defense/resistance mechanisms. To ease understanding,
182 hereafter resistance is used for large increases in MIC values and tolerance for substantial increases
183 primarily in MBC values without the MIC necessarily changing.

184 Changes in **silver and copper surface tolerance** of the evolved populations and selected mutants were
185 tested similarly to exposures in evolution experiments. Surfaces used and exposure times for testing
186 populations were identical to respective evolution experiments. For testing mutants unused metal
187 surfaces were employed to reduce variability but as the unused surfaces are less efficient than
188 repeatedly used and cleaned ones, exposure times were changed to: 6 h on copper and silver in high-
189 organic humid conditions, 35 min on copper and 4 h on silver in low-organic dry conditions.
190
191 To determine **minimal inhibitory concentrations (MIC)** of ionic silver and copper as well as selected
192 antibiotics of the evolved populations and selected mutants compared to the ancestral strain of *E. coli*
193 ATCC 8739 a modified standard agar dilution method [44] was used . Briefly, stock dilutions of
194 respective soluble metal salts or antibiotics were suspended in 50°C Mueller-Hinton agar (MHA; Biolife
195 MH Agar II #4017402; Biolife Italiana, Milan, Italy) in 10x10 cm square Petri dishes. To prepare the
196 inoculum by least hindering mutant proportions in the evolved populations and avoiding competition in
197 liquid culture, the evolved populations and the ancestral inoculum material were streaked onto LB agar
198 from glycerol stock and incubated at 37°C for 24 h. The resulting biomass was suspended in 5 ml PBS
199 and pipetted into clear 96-well plate in triplicates. All the suspensions were adjusted to 10^7 CFU/ml
200 photometrically after which 3 µl spots ($3x10^4$ CFU) of each suspension was inoculated onto MHA plates
201 supplemented with different target substance concentrations. The results were registered as presence
202 or absence of visible growth after 24, 48, 72 and 168 h of growth to account for possible mutants with
203 higher tolerance but lower growth rate. Only data from 24 and 48 time points are demonstrated as the
204 results did not change with longer incubations.
205
206 To determine **minimal biocidal concentration (MBC)** of ionic silver and copper of the populations
207 evolved on the respective metal surfaces, a drop test protocol from Suppi et al. [45] was used with
208 minor modifications. Shortly, bacterial suspensions in deionized water or organic soil load were exposed
209 to 2x dilution series of silver and copper salts at room temperature in static conditions 5 µl of which was
210 drop plated to non-selective solid LB for MBC determination after 2 h and 24 h of liquid exposure. In the
211 tests with evolved populations, bacterial suspensions from plate cultures (as described for evolution
212 experiments) were used instead of liquid culture to least affect the mutation frequency pattern of the
213 population. In the tests with selected mutants, an exponential liquid LB culture was used as described in
214 Suppi *et al.* [45].

215

216 **Assessment of dihydrogen sulfide production**

217 To assess H_2S production in the SL medium from the high-organic humid exposure conditions (Table 1),
218 colorimetric test based on formation of dark insoluble metal sulfides was used. For that SL was
219 supplemented with 0,5 g/l $(NH_4)_5[Fe(C_6H_4O_7)_2]$ as the metal ion source, 4 g/l K_2HPO_4 as a buffering agent,
220 and 1,94 g/l L-cysteine as the sulfur source. Formation of dark insoluble sulfides was registered
221 qualitatively by visual inspection after incubation at 37°C for up to 24 h.

222 **Sequencing and data analysis**

223 The initial inoculum from the 1st cycle and harvested biomass from the 30th cycle of both evolution
224 experiments was frozen at -20°C until DNA extraction with Invitrogen™ PureLink™ Microbiome DNA
225 Purification Kit (Thermo Fisher Scientific; #A29790). DNA samples were sequenced by Novogene Co. Ltd
226 using Illumina NovaSeq 6000 resulting in 150 nt paired end reads with ~368±167-fold genomic coverage
227 for *E. coli* ATCC8739 out of which >98% of the sequences per sample mapped to the reference genome
228 (GenBank: CP000946.1). Raw reads were deposited to the European Nucleotide Archive (ENA), accession
229 PRJEB75034. Breseq analysis pipeline was used for mutation analysis in polymorphism mode [46], [47].
230 Mobile genetic elements in the reference sequence of the ancestor strain were detected by ISfinder
231 [48].

232 **Statistical analysis**

233 In GraphPad Prism 9 software one-way or multifactorial ANOVA with multiple comparisons at α=0.05
234 was used to determine statistical significance of the quantitative results. CFU values were $\log_{10}$
235 transformed prior to analysis.

236

237 **Results and discussion**

238 **Experimental system**

239 The evolution experiments consisted of 30 cycles of exposure of *E. coli* ATCC 8739 on copper, silver,
240 steel or glass surfaces in 5 parallel lineages for exposure durations shown on Fig. 2. Between exposures
241 the survivors were clonally grown on solid medium to accumulate mutations and prevent selection for
242 growth rate in liquid medium as mutations that increase copper surface tolerance have been shown to
243 cause slower growth[32].

244 To address the variable efficacy of copper and silver surfaces in different application-relevant conditions
245 [2], growth supporting high-organic humid (HH) and non growth supporting bactericidal low-organic dry
246 (LD) exposure conditions were selected for the cyclic exposure experiments as detailed in Table 1. Due
247 to different efficacy of copper and silver surfaces in LD and HH conditions, different exposure duration
248 had to be determined. Exposure time on control surfaces was selected according to longest exposure on
249 antimicrobial surfaces in the same exposure conditions to maximally reflect selection due to exposure
250 conditions. The conditions selected for LD experiment aimed for an about 2 $\log_{10}$ reduction in viable
251 counts compared to initial inoculum on antimicrobial copper and silver surfaces which also caused a 1.5
252 $\log_{10}$ reduction on glass and steel control surfaces (Fig 2A). In pilot experiments, larger reduction on

253 antimicrobial surfaces achieved by longer exposures further increased variability of viable counts and by
254 that could have complicated assessment of changes in surface tolerance as well as led to loss of lineages
255 in evolution experiments. The conditions in the HH experiment allowed up to 320% growth on glass
256 surfaces during 6 h, less on steel and no growth on silver surfaces (Fig 2B). Copper surfaces
257 demonstrated substantial antibacterial activity already after 1 h exposure. Therefore, shorter exposure
258 was used on copper in HH conditions.

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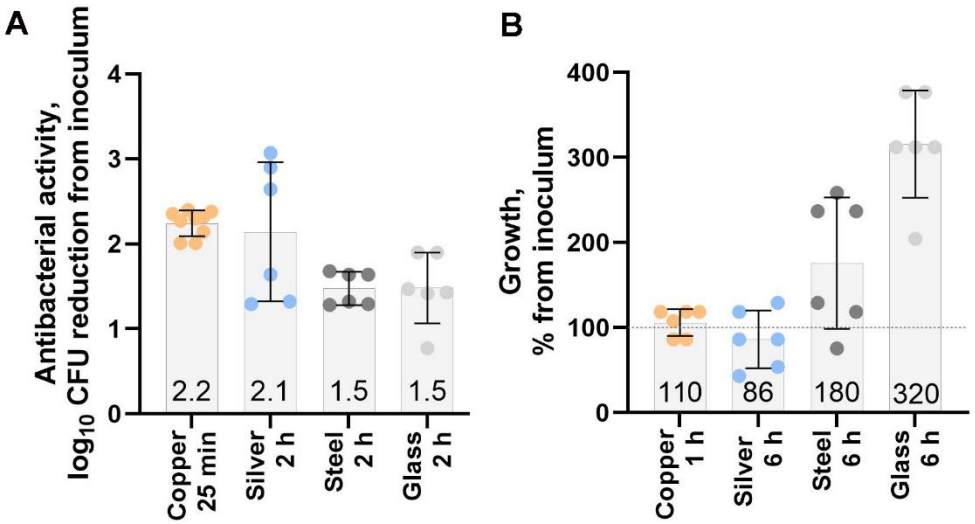


260 **Figure 2. Initial antibacterial activity of the surfaces in low-organic dry (LD) conditions (A) and**
261 **bacteriostatic effect of metal surfaces in high-organic humid (HH) conditions (B)** against the ancestral *E.*
262 *coli* ATCC 8739 strain in evolution experiment conditions. Mean values from 3 independent experiments
263 of antibacterial activity ($\log_{10}$CFU/surface in inoculum – $\log_{10}$CFU/surface post-exposure) (A) and % growth
264 from the inoculum (B) ±SD are presented on respective bars on graph.

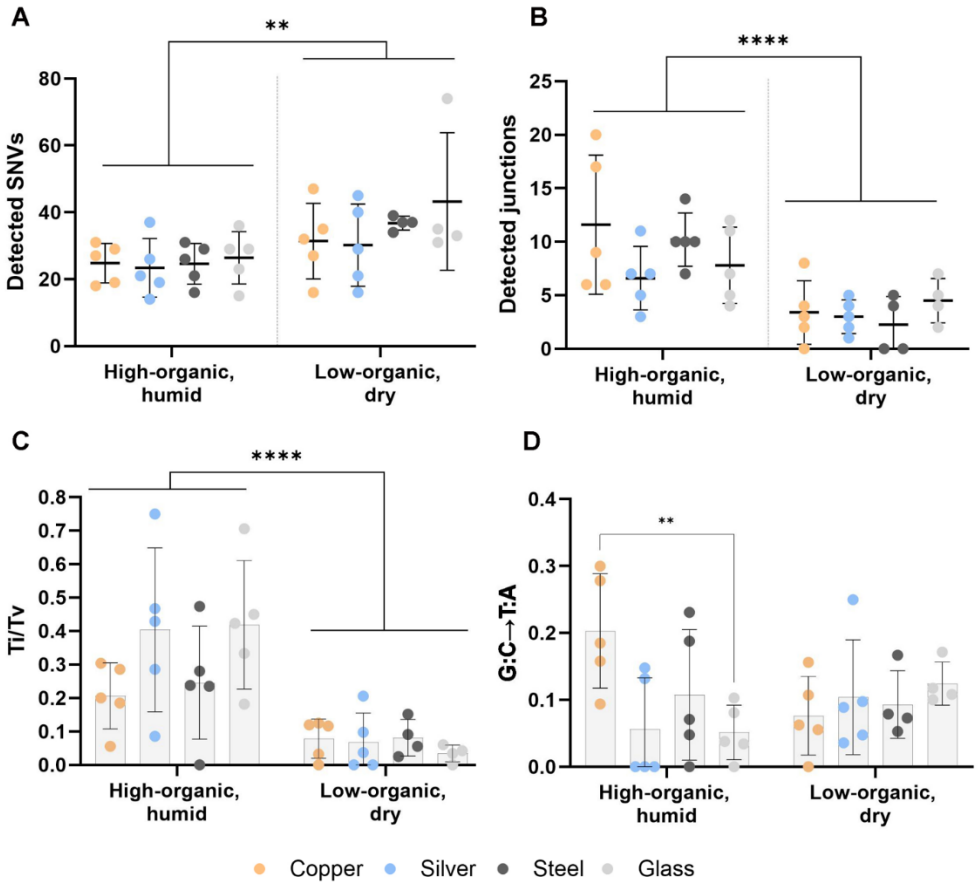


Figure 3. Types of mutations detected in the evolved populations. A - total number of single nucleotide variations (SNVs); B – total number of larger genomic rearrangements (new junctions); C – proportion of transitions (Ti) to transversions (Tv) among the detected SNVs; D – guanine to thymine substitution proportion among the SNVs detected in the WGS data of the evolved populations from high-organic humid (HH) and low-organic dry (LD) exposure conditions at ≥0.05 frequency after removing mutations present in the ancestor. There were no significant differences in the number of SNVs (A), junctions (B) or transitions to transversions ratio (C) accumulated during the evolution on metal surfaces compared to the control evolved on glass in the same conditions. Significantly higher number of SNVs were detected in LD exposure conditions compared to HH in general with the exposure environment explaining 23% of total variation in SNV counts and 49% of total variation in transitions to transversions ratio based on two-way ANOVA with post-hoc testing for multiple comparisons. The environment of the evolution experiments did not contribute to variation of G->T substitutions (D) but compared to glass more G->T transversions were accumulated on copper in HH conditions. Several larger genomic arrangements were detected (B), usually involving IS element mobility and rDNA reorganization, but as not all such events could be fully annotated using short-read data, total number of new junctions detected is presented. Significantly higher number of junctions were detected in HH exposure conditions compared to LD in general with the exposure environment explaining 41% of total variation in junction counts based on two-way ANOVA with post-hoc testing for multiple comparisons. The ancestral inoculum of the HH experiment also contained more rDNA reorganization events compared to the reference genome than the one of the LD experiment and could lead to a random bias. However, the same association held after removing all rDNA reorganizations from the comparison, with exposure environment still explaining 20% of total variation (Supplemetaty Fig. S1). * P< 0.05; ** P< 0.01; *** P< 0.001; **** P< 0.0001

288 **Mutation frequency and single nucleotide variations**

289 In whole genome sequences of populations evolved on copper, silver or steel surfaces no significant
290 differences in the number of accumulated single nucleotide variants (SNVs) compared to populations
291 evolved on glass were found (Fig 3A). This result opposes the finding by Xu et al. [32] that demonstrated
292 elevated mutation accumulation in *Pseudomonas fluorescens* on copper surfaces. Although Xu et al.
293 explained the high mutation frequency with mutated *mutL* in copper-exposed lines, they also had a
294 mutated *mutS* in LB-passaged controls indicating that the evolution experiment might have been carried
295 out with a mismatch-repair impaired inoculum inherently elevating mutation rate. There were no
296 mismatch-repair system mutations present in copper or silver exposed lineages in our results. The
297 conflicting results highlight the need to sample and sequence the material from the first inoculum of the
298 evolution experiment.

299 Among the detected SNVs, there were no significant differences in the ratio of transitions to
300 transversions in the populations evolved on metal surfaces compared to glass (Fig 3C). However, general
301 higher than the expected(*e.g.* 44% transversions in wild-type *E. coli* MG1655 [49]) transversion
302 accumulation not contributable to oxidation-prone G>T substitution (Fig 3D) but rather A:T>T:A
303 (Supplementary Fig. S2) was unexpected. Similar tendency on all surface types suggests a strain-specific
304 effect. Unexpectedly high presence of transversions and even higher transversion proportion in LD
305 conditions than HH conditions could indicate nutrient limitation [50] and stronger purifying selection
306 [51]. High level of T:A>A:T substitutions is scarcely described in previous studies but has been indicated
307 in highly transcribed regions [52] or in case of specific mutations *e.g. recA*730 (E38K) [53]. Higher
308 proportion of G->T substitutions was found on copper compared to glass in high-organic humid
309 conditions (Fig 3D) possibly indicating oxidative damage of guanine [54], [55]. Reactive oxygen species
310 (ROS) damage on copper surfaces is an expected mechanism due to copper [56] and iron [57] producing
311 Fenton-like reactions unlike silver and glass.

312

313 **Genomic rearrangements**

314 Larger genomic rearrangements (Fig. 3B), especially IS1-mediated deletion enrichment was abundantly
315 observed after exposure to control surfaces in high-organic humid conditions where bacteria were
316 proliferating also during surface exposure and were much less frequent, but not missing, on
317 antimicrobial surfaces in low-organic dry conditions (Fig 3B, Table 2, Supplementary Table S1). The initial
318 inoculum of the high-organic humid experiment also contained more rRNA reorganizations than the one
319 for LD experiment. The cause of this variation as well as its possible effect on evolutionary scenarios
320 remains unknown.

321 IS element presence in “safe strains” is well known [58]. In the genome of *E. coli* ATCC 8739 forty-eight
322 IS elements from eight IS families were detected with IS1 elements being the most abundant
323 (Supplementary Fig. S3).

324

325 Deletions directly adjacent to existing IS1 elements could possibly be explained by abortive transposition
326 of the IS resulting in flanking deletions of variable length [59]. Similar phenomenon has been recorded
327 for example in previous *E. coli* evolution experiments that have resulted in loss of motility due to

328 deletions in flagellar genes next to an IS1 element [60] and in case of deletions next to IS1X2 element
329 causing adaptive nitrofurantoin resistance in pathogenic *E. coli* [61]. Although higher IS mobility in *E. coli*
330 ATCC 8739 has not been experimentally proven, such deletions could be more frequent in the presence
331 of insertion sequence-excision enhancer (*iee*) protein [62], [63] with the helicase domain highly similar
332 to *EcolC_1463* (annotated as *radD*) of *E. coli* ATCC 8739. Furthermore, *E. coli* ATCC8739 has an IS1X2
333 insertion into *clpA* (substrate-specifying adapter for the ClpAP protease complex) reading frame and
334 disrupted protein degradation has been shown to lead to more frequent IS excision [64]. IS1X2 is also
335 found to be significantly associated with antibiotic resistance genes (within 10 ORF distance) in
336 metagenomic datasets [65].

337 Our results showed that IS1X2 located at *EcolC_2714* in the reference genome seems to be especially
338 prone to cause deletions of variable length starting with *EcolC_2715.* These deletions were detected in
339 different exposure conditions all spanning at least the reading frames of *EcolC_2715*(*clpS*) and
340 *EcolC_2716*(*cspD*) with the longest deletion extending to *EcolC_2749*. Deletions with different lengths
341 can be present in the same evolved population at high frequencies. Universal benefit of the deletions is
342 not directly clear but could be speculated to be associated with *cspD* replication inhibitor inactivation
343 rather than *clpS* because the *EcolC_2714* IS1 itself is an insertion into *EcolC_2713*(*clpA*) expected to
344 inactivate the ClpSAP-dependent protein degradation pathway. Excess of CspD is toxic to cells [66]. *CspD*
345 is regulated by the MsqRA toxin-antitoxin system [67] which responds to stress with antitoxin MsqA
346 degradation by ClpXP followed by *cspD* derepression. *CspD* deletion could avoid its increased toxic levels
347 independently from stress-responsive regulation. CspD has also been associated with persister
348 formation [68]. Deletion of *cspD* and its next neighbor efflux system encoding *macAB* could therefore
349 indirectly be considered decreasing the probability of antimicrobial resistance development and spread
350 but are clearly dependent on strain specific IS1 location.

351

352

Table 2. Mutations accumulated in populations evolved on copper and silver surfaces in low-organic dry (LD), and high-organic humid (HH) exposure conditions at ≥0.20 frequency. Grey numbers indicate mutation frequency <0.20 in cases where mutations affecting the same gene also occur at ≥0.20 frequency in other populations. Grey background marks the genes with mutations accumulated also on control surfaces. Synonymous SNVs, rRNA reorganizations and intergenic changes downstream of neighboring reading frames are excluded from the table.

				Ag_LD					Ag_HH					Cu_LD					Cu_HH				
Position	**Annotation**	**Affected loci**	**Affected genes by similarity**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
1,584,799	Y343* (TAC→TAG)	EcolC_1435	*ompC*	1.0																			
4,430,894	E145D (GAA→GAT)	EcolC_4022	*zraS (hydH)*	0.3																			
825,348	F231V (TTT→GTT)	EcolC_0772	*metK*	0.3	0.3	0.3			0.2				0.2					0.2	0.2		0.2		
3,450,094	Q19K (CAG→AAG)	EcolC_3147	*apt*		0.4																		
2,196,802	intergenic A→T (-3)	EcolC_1988	*slyB*			0.9																	
	intergenic A→T (-271)	EcolC_1989	*anmK*			0.9																	
2,629,020	::IS1 (1714-1723/2676 nt)	EcolC_2387	*adhE*			0.6																	
2,774,065	A97P (GCG→CCG)	EcolC_2521	*flgH*				0.5																
1,472,947	intergenic C→T (-185)	EcolC_1342	*argT*					0.5															
1,755,361	intergenic T→A (-47)	EcolC_1578	*yegH*					0.8															
	intergenic T→A (-612)	EcolC_1579	*wza*					0.8															
2,987,581	Δ 2,440 bp	EcolC_2715-EcolC_2717	*clpS,cspD,...*											0.9									
3,551,945	::IS1 (271-279/678 nt)	EcolC_3242	*aroM*												0.9								
2,634,829	V120F (GTT→TTT)	EcolC_2392	*rssB*													1.0							
3,111,116	G13R (GGG→AGG)	EcolC_2843	*ybiB*													1.0							
2,987,583	Δ 32,016 bp	EcolC_2715-EcolC_2745	*clpS,cspD,...*														1.0						
784,740	::IS1 (167-174/645 nt)	EcolC_0730	*nemR*-like														0.9						
1,263,419	T986I (ACA→ATA)	EcolC_1157	*yfhM*														1.0						
1,049,782	Q440* (CAG→TAG)	EcolC_0961	*cysN*															1.0					
4,265,419	::IS1 (269-277/3360 nt)	EcolC_3887	*EcolC_3887*															1.0					
4,740,468	M266I (ATG→ATT)	EcolC_4286	*tnaA*						0.4	0.3	0.4	0.7	0.1										
2,105,539	::IS1 (209-217/1392 nt)	EcolC_1903	*ydjN (tcyP)*							0.2													
2,105,625	G44D (GGC→GAC)									0.1													
2,666,933	intergenic T→C (-117)	EcolC_2421	*ychH*								0.2												
	intergenic T→C (-161)	EcolC_2422	*pth*								0.2												
2,987,582	Δ 1,037 bp	EcolC_2715-EcolC_2716	*clpS,cspD*								0.1												
2,987,583	Δ 8,906 bp	EcolC_2715-EcolC_2722	*clpS,cspD,...*								0.2												
2,987,583?	Δ 7,339 bp	EcolC_2715-EcolC_2720	*clpS,cspD,...*									0.4											
2,987,583?	Δ 6,100 bp	EcolC_2715-EcolC_2720	*clpS,cspD,...*																		0.2		
88,398	S245C (AGT→TGT)	EcolC_0083	*rfaJ*																		0.2		
465,883	intergenic 53 bp→54 bp (-189)	EcolC_0434	*yrdA*																	0.3			

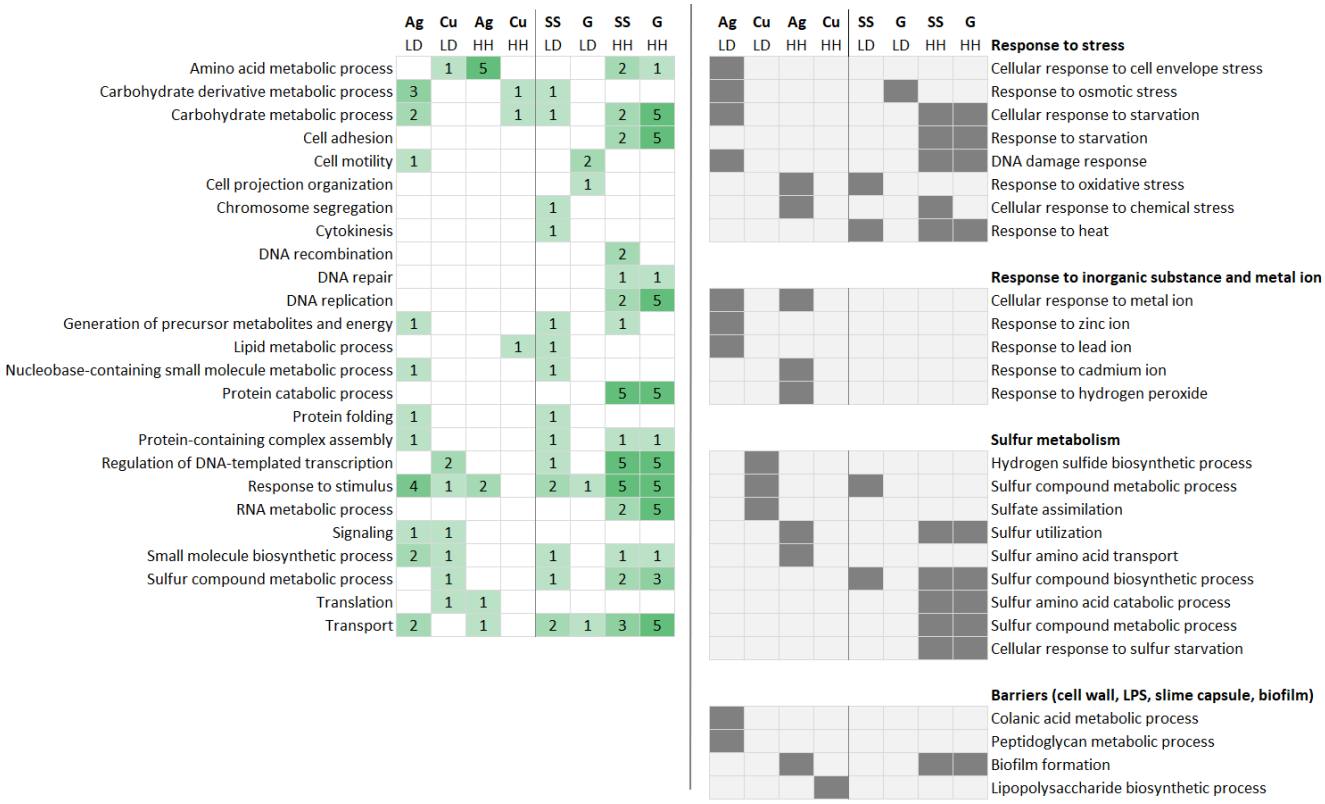


Figure 4. Functional annotation of genes potentially affected by mutations accumulated with ≥0.2 frequency in population evolved on silver, copper, stainless steel (SS) and glass (G) in low-organic dry (LD) and high-organic humid (HH) exposure conditions. Number of parallel lineages where mutations with shared GOslim_prokaryote annotations are presented in green gradient (left panel) and presence of more specific GO terms is presented as dark squares (right panel). QuickGO [69] was used to filter Gene Ontology database [37]. For copper and silver, occasionally occurring IS1-mediated deletions (*EcolC_2715-…*) also abundantly present on control surfaces were excluded from the analysis.

Specific mutation accumulated on silver and copper surfaces at over 0.2 frequency in evolved populations are presented in Table 2. While trying to connect functional annotations (Fig. 4) of the mutated genes in the populations to their role in (metal) stress tolerance, one must keep in mind that not all mutations in a population might have been selected for. Random mutations in the same genome with a beneficial mutation enrich in frequency just by genetic linkage ("hitchhiking").

Mutations: metal sensing and heavy metal homeostasis

E. coli ATCC 8739 contains chromosomal ***pco*** (*EcolC_3410-3417*) copper resistance and acquisition [70], [71], [72] as well as ***sil*** (*EcolC_3420-3428*) silver resistance [36], [73], [74] gene clusters forming a heavy metal homeostasis/resistance island [34]. The whole *EcolC_3410-EcolC_3428* region shows 97% sequence similarity to the historical metal resistance plasmid pMG101 (GenBank: NZ_JAFFIC010000003.1) and could be of possible plasmid origin via an independent transposition event [75], [76]. Surprisingly, no changes in the *sil* genes of the silver-exposed lines were detected after the evolution experiment although importance of such mutations in increased silver tolerance in addition to the mere presence of the *sil* genes have been described [35], [77]. No mutations in known copper

383 uptake or resistance genes were also detected in populations evolved on neither copper nor silver
384 surfaces.

385 **ZraS** E145D in histidine kinase of the ZraSR two-component envelope stress signaling pathway that was
386 enriched in Ag1_LD population could be a hitchhiker to a fixed OmpC Y343* in the same population but
387 could also plausibly contribute to metal tolerance. The observed amino acid substitution occurs in the
388 periplasmic sensor-like domain (InterPro: P14377 [78]), not the kinase domain. Mutations in sensory
389 domain of ZraS might change sensor affinity to other metals besides the already known Zn and Cu [79].
390 ZraS is a member of the atypical two-component regulatory system ZraSR. ZraS functions as a
391 membrane-associated protein kinase that phosphorylates ZraR in response to high concentrations of
392 zinc or lead in the medium resulting in expression of periplasmic metal-binding chaperon ZraP which
393 simultaneously acts as a repressor of ZraPSR [79], [80]. ZraR can also regulate expression of other stress
394 response genes important in maintaining membrane integrity in the presence of biocides targeting the
395 envelope [81]. Surprisingly, also *e.g.* similarly to PspF, CpxR and sigma E in mutant studies [82] in the
396 presence of Zn^{2+} ZraR strongly downregulate expression of the indole and H_2S producing tryptophanase,
397 *tnaA* [81]. However, tryptophanase activity might be protective in the presence of silver (see below).
398 The exact role of the metal responsive ZraPSR in copper and silver response is not clear while the main
399 role might be associated with envelope stress response similarly to the Cpx system and not metal
400 homeostasis.

401 **Mutations: cell surface and membrane transport**

402 The outer membrane proteins **OmpC** and OmpF, are the two major porins of *E. coli*. Although cross-
403 membrane transport and tolerance mechanisms of silver and copper ions in bacteria partly overlap, it is
404 known for decades that conditions selecting for silver tolerance might not by default also grant
405 increased copper tolerance [5]. Similarly to *zraS* carrying mutation in the same silver-exposed
406 population, OmpC is also indicated in the Gram-negative envelope stress response via CpxAR-regulation
407 [83].

408 The role of outer membrane porins in metal tolerance is somewhat unclear. For example, it has been
409 demonstrated that the lack of *ompC* and/or *ompF* alone does not seem to grant significantly increased
410 silver and/or copper salt MICs in *E. coli* laboratory strains [5] while deletion of one or both of the genes
411 from *E. coli* MC 4100 increased tolerance to both silver ions and nanoparticles [84]. It can also be that
412 while defective porin function alone is not enough then mutations in both passive influx by Omp porins
413 and active efflux by Cus can increase silver tolerance of *E. coli* [33], [36], [73]. Complexity and
414 differences in transcriptional regulation and presence or absence of functional homologues in different
415 *E. coli* strains might influence individual porin role in metal tolerance.

416 There seems to be confusion about the *ompC* genes in the published *E. coli* genomes. The *ompC* gene
417 seems to have undergone substantial mosaic evolution [85] and *ompC* of *E. coli* ATCC 8739 has been
418 previously described to be disrupted by an IS1 insertion [86]. Surprisingly, neither o*mpC* (*EcolC_1435*) of
419 the *E. coli* ATCC 8739 reference genome (CP000946.1)[86] nor respective genes in other accessible ATCC
420 8739 genomes (CP022959.1; CP033020.1) had a truncated *ompC* and instead showed 91.17% similarity
421 with 100% query coverage when BLASTed against full length *ompC* (*b2215*) of the *E. coli* K-12
422 (U00096.3). *E. coli* ATCC 8739 sequence CP043852.1 seemed to have a 100% similar *ompC* to K-12,
423 lacked the *pco* and *sil* gene clusters and the genome might be wrongly designated to ATCC 8739 strain.
424 The reference genome also contains an unannotated intact *micF* regulatory RNA sequence. Genomic

425 sequences of several *E. coli* B strains, however, did present a truncated *ompC* as has also been reported
426 in the literature [87], [88]. Resequencing of *the E. coli* ATCC 8739 ancestor strain revealed no deletions
427 or IS insertions in *ompC*. Therefore, we expected *E. coli* ATCC 8739 to have a full length functional *ompC*.
428 Furthermore, we detected a fixed mutation of a premature stop codon (Y343*) in *ompC* in the silver-
429 exposed Ag1_LD line indicating selective pressure towards *ompC* inactivation.

430 ***YfhM*** with a fixed mutation in Cu4_LD is a colonization factor, an α_2-macroglobulin that traps host
431 proteases and works in close association with peptidoglycan glycosyltransferase acting as a as a defense
432 and repair mechanism [89], [90]. The biological effect of substitution T986I is located between the bait-
433 region and thioester domains in one of the several macroglobulin-type domains remains unclear but
434 could be associated with cell wall repair. Copper toxicity, indeed, has been associated with envelope
435 stress, impaired lipoprotein maturations and peptidoglycan cross-linking [91].

436 Other enriched mutations potentially affecting outer barrier of the cell were between the genes *slyB* (-3)
437 and *anmK* (-271) in Ag3_LD, between *yegH* (-47) and *wza* (-612) in Ag5_LD and *rfaJ* S245C in Cu3_HH.
438 ***SlyB*** is a stress-induced PhoPQ-regulated outer membrane lipoprotein that is essential in outer
439 membrane stabilization [92] and is associated with antimicrobial peptide and polymyxin B tolerance
440 [93], [94], [95] while ***AnmK*** participates in peptidoglycan recycling [96]. ***YegH*** encodes a putative inner
441 membrane transporter immediately neighboring the colonic acid transport and synthesis genes starting
442 with ***wza*** in the opposite direction[97]. ***RfaJ*** participates in LPS synthesis [98].

443 However, the potentially cellular barrier function affecting mutations alone were generally not enough
444 to substantially increase copper surface tolerance of the evolved populations carrying the mutations (Fig
445 5).

446 **Mutations: sulfur metabolism**

447 Mutation in several genes associated to sulfur metabolism or sulfur amino acid transport and
448 metabolism were detected in copper and silver exposed populations mostly in high-organic humid
449 conditions.

450 M266I substitution in tryptophanase (*EcolC_4286,* ***tnaA***) was enriched in all silver-exposed lineages
451 (frequency 0.13-0.70) in high-organic humid exposure conditions and not on any other surface or in low-
452 organic dry conditions. Contribution of adaptive *tnaA* mutations to increased stress tolerance has been
453 described *e.g.* for heat shock but the underlying mechanisms can be challenging to interpret [99]. TnaA
454 and OmpC from soluble fraction of *E. coli* proteome have been shown to be bound to and TnaA also
455 inactivated by AgNPs [100]. AgNPs are well known to act via release of ionic Ag similarly to silver
456 surfaces. In the case of M266I, the conserved Ag-vulnerable methionine is substituted with structurally
457 similar but less vulnerable isoleucine in close proximity of pyridoxal-phosphate modified K270 in the
458 active center of the tetrameric enzyme [101]. Therefore, we assume that the mutation could protect the
459 enzyme function under silver stress. Tryptophanase is best known for degrading exogenic L-tryptophan
460 resulting in production of indole (L-tryptophan + H_2O => Indole + Pyruvate + NH_3) but participates in
461 detoxifying excess amounts of exogenous L-cysteine, is highly inducible by L-cysteine and well known to
462 catalyze reactions leading to hydrogen sulfide production (L-cysteine + H_20 => Pyruvate + NH_3 + H_2S and
463 Indole + L-cysteine => L-tryptophan + H_2S) [102], [103], [104]. Furthermore, out of the several enzymes
464 capable of H_2S production in *E. coli* [103], [105], [106], TnaA can be the main producer in rich medium
465 [107]. Signaling and stress-related role of H_2S in bacteria is poorly described but increased H_2S

466 production has been previously associated with increased silver tolerance of *E. coli* [108], alleviating
467 heavy metal toxicity to fungi [109] and offering protection against oxidative stress and antibiotics [110].
468 Similarly, periplasmic H_2S-dependent biomineralization of cadmium has been demonstrated in *E. coli*
469 [111]. *E. coli* ATCC 8739 indeed produces both indole[112] and hydrogen sulfide (Supplementary Fig.
470 S4). While indole can play a role in stress signaling inside and between bacterial cells hydrogen sulfide
471 can freely cross bacterial membranes by passive diffusion and potentially directly detoxify intracellular
472 and extracellular silver ions by forming insoluble silver sulfide. TnaA expression is downregulated by
473 glucose and metal (II) ions and upregulated by oxidative stress, high pH and temperature [113]. On the
474 other hand, inactivation of TnaA and thereby at least partially blocking L-cysteine degradation could
475 theoretically direct cysteine to glutathione production by GshF [114] and help with intracellular, but not
476 extracellular Ag detoxification.

477 In Ag2_HH lineage with relatively lower TnaA M266I frequency (0.29) we also observed IS1X2 insertion
478 into EcolC_1903 (***ydiN;*** 0.21) reading frame and G44D substitution in the same gene (0.12) possibly
479 indicating competition of beneficial mutations in the population. *YdjN* encodes one of the two cystine
480 importers in *E. coli* [115], [116] which can import cystine in oxic conditions. YdjN is required by *E. coli* for
481 growth on S-sulfocysteine as a sulfur source [117], has been demonstrated to contribute to oxidative
482 stress tolerance [118], and its expression can be upregulated by silver nanoparticle exposure [119].
483 However, defective YdjN -mediated transport has been observed in case of *E. coli* resistant to toxic
484 selenocystine [115]. In our case possible *ydjN* inactivation could avoid uptake of Ag-cysteine complexes
485 in high-organic conditions in the presence of peptides and amino acids free to react with silver in the
486 extracellular space.

487 The Cu4_LD population with a fixed mutation in ***cysN*** (Q440*) also carries an IS insertion into
488 ***EcolC_3887*** with unknown function. Up-regulation of cysteine biosynthesis genes, including *cysN*, has
489 been indicated in response to metal ions above physiological concentrations, *e.g.* zinc [120] and copper
490 [121]. Counterintuitively, we observed a fixed premature stop codon in *cysN* in the population evolved
491 on copper in low-organic dry conditions. CysN is one of the first enzymes in cysteine biosynthesis
492 pathway by activating sulfate in sulfur assimilation process. CysN upregulation in response to heavy
493 metal exposure could be an indirect result of elevated sulfide levels triggering sulfide detoxification to
494 sulfite and sulfate [122], [123]. In the presence of organic sulfur sources, interruption of this
495 detoxification process by affecting sulfide-sulfate balance could theoretically lead to toxic levels of
496 intracellular sulfide that could, although at a price, detoxify the metal ions. However, emergence of the
497 mutation in low-organic dry conditions complicates the logic.

498 Finally, ***yrdA*** with an upstream (-189) mutation in Cu2_HH seems to be encoding a cytosolic Zn^{2+}-binding
499 hexapeptide repeat acetyltransferase, gamma carbonic anhydrase like protein with unknown function
500 that is induced by polyamine and azide [124], [125], [126]. Interestingly, *yrdA* shared expression pattern
501 with *tnaA* in polyamine exposure conditions [125] and in *Bacillus subtilis yrdA* is possibly associated with
502 assimilatory sulfate reduction [127].

503 The environment of the high-organic humid experiment could explain some of the context of mutations
504 accumulated that were associated with sulfur and amino acid metabolism. The high-organic soil load
505 (2.5 g/L BSA, 3.5 g/L yeast extract, 0.8 g/L mucin) is rich in protein with strong overrepresentation of
506 sulfur-containing amino acid cysteine, the substrate of TnaA for H_2S production and transport target for
507 YdjN. There are 35 cysteines out of 607 amino acids (5.8%) in BSA (Uniprot: P02769) and 118 cysteines

508 out of 1390 amino acids (8.5%) in mucin (Uniprot: A0A5G2QSD1) compared to around 1% in *E. coli* in
509 general [128]. Serum albumins are also well known to bind metal ions, metal complexes and metallic
510 nanoparticles, including those of copper and silver [129], [130], [131] which causes structural changes in
511 the protein and Cu(II) specifically causes BSA to aggregate [130]. Mucin is also known to act as a copper
512 chaperone that binds copper in both its Cu(I) and Cu(II) forms [132]. The high cysteine content of the
513 organic soiling medium could partly explain enrichment of sulfur metabolism mutations and should be
514 considered while comparing the results to other exposure media.

515

516 **Mutations: motility and biofilm**

517 Unfortunately, due to presumably isogenic single-colony origin of the ancestral culture of the HH and LD
518 evolution experiments individually we ended up having a fixed (0.90) mutation in the ancestor of the HH
519 experiment rendering the bacteria non-motile by introducing an early stop codon (W8*; TGG→TGA) in
520 *fliI* (*EcolC_1701*) gene that codes an ATPase involved in flagellar assembly. As other motility-impairing
521 mutations were also detected on control surfaces and there seemed to be no selection against the FliI
522 W8* we concluded that the mutation did not substantially affect metal tolerance. Motility is highly
523 energy-expensive [133] and loss of the function could be energetically beneficial in clearly resource-
524 limited conditions. Additionally, the loss of function by an immature stop codon proved to be easily
525 reversable as we repeatedly saw in the motility assay (data not shown).

526 **Other high-frequency mutations**

527 Cu3_LD population carried stress response associated fixed mutations of V12F in response regulator
528 domain of *rssB* and G13R in glycosyl transferase domain of *ybiB.* **RssB** is a σ^S stability modulator in *E. coli*
529 [134] absence of which increases σ^S stability and bacterial osmotic, oxidative and heat stress tolerance
530 [135]. **YbiB** is a poorly characterized LexA-regulated DNA-binding protein that might be involved in SOS
531 response and persistence [136] interruption of which is associated with decreased fitness in response to
532 metal toxicity (ROS-active CrO_4^{2-}) of metal tolerant *Pantoea sp*. MT58[137].

533 It is not immediately clear how mutations in adenine phosphoribosyltransferase ***(apt)*** could increase
534 silver surface tolerance, but Q19K substitution N-terminal from the phosphoribosyltransferase domain
535 of the encoded protein seems to increase viability of Ag2_LD population on silver surface (Fig 5). If the
536 mutation impairs purine salvage and adenine is not converted to AMP by apt or the reaction is less
537 efficient, one could suspect that as adenine has high affinity towards silver ions [138], [139], some of the
538 adenine that is not recycled or is processed by Apt less efficiently could in theory participate in silver
539 detoxification intracellularly and possibly also extracellularly. Disruption of *apt* has also been shown to
540 cause reprogramming of nucleotide metabolism, adenine accumulation and increased copper tolerance
541 while not affecting intracellular copper accumulation in *S. aureus* [140]. Furthermore, overexpression of
542 not only *apt* but also other enzymes that use phosphoribosyl diphosphate (PRPP), sensitized *cop*$^-$ strain
543 of *S. aureus* to Cu^{2+} indicating a more general involvement of Cu^{1+} induced blocking of the pentose
544 phosphate pathway and sparing of PRPP protecting against Cu^{2+} toxicity. As toxicity, transport and
545 detoxification of Ag^{1+} largely overlap with Cu^{1+} it is plausible that possible impairment of *apt* offers
546 protection against both.

547 **Phenotypic characterization of evolved populations: metal surface tolerance**

548 To best describe the adaptational benefits of the accumulated mutation patterns in the evolved
549 populations, silver-exposed populations were exposed to silver surfaces and copper-exposed
550 populations to copper surfaces in the evolution experiment conditions (Fig 5). In high-organic humid
551 conditions the populations harboring higher frequency of mutated *tnaA* tended to have a slight
552 advantage over the ancestor (Fig 5A). However, as this advantage is growth-limited in already sub-
553 optimal growth conditions (Fig. 2B), which means that compared to potentially large changes in
554 mortality in bactericidal conditions (Fig. 2A) the potential growth benefit is expected to be small. Despite
555 methodological challenges in detecting small changes in phenotypic traits, even a slight advantage in
556 fitness may become biologically relevant. No changes in metal surface tolerance were observed in the
557 case of copper-exposed surfaces in high-organic humid conditions, probably due to low biocidal and/or
558 growth-related selection pressure.

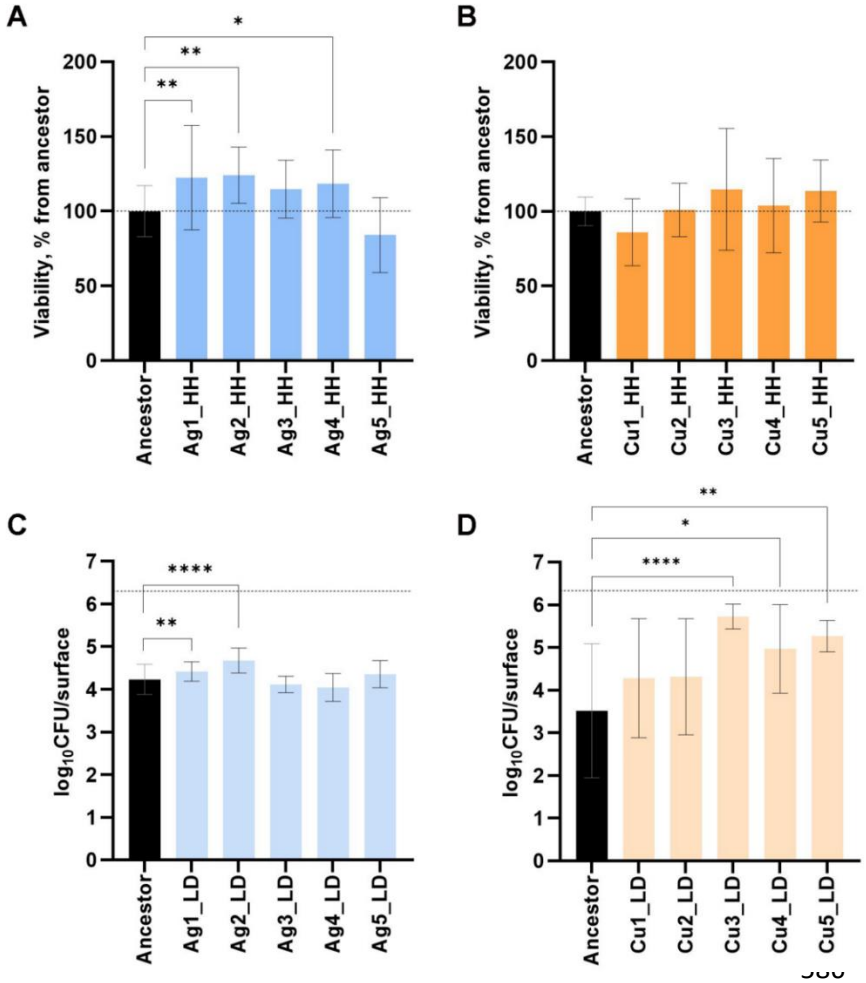


Figure 5. Silver and copper surface tolerance of the evolved communities. Communities evolved in high-organic humid (A,B) or low-organic dry (C,D) environments exposed to copper (B, D) or silver (A,C) surfaces were tested against the ancestral strain in the same conditions as in the corresponding evolution experiment. In growth-supporting HH exposure conditions (A,B) relative change in post-exposure viable counts compared to ancestor is presented. Black dotted line indicates ancestral baseline. In bactericidal LD exposure conditions (C,D) post-exposure viable counts are presented with a reference to initial viable count in the inoculum (black dotted line. Values from three independent experiments are presented with mean ±SD. Statistical significance of the difference from the ancestor exposed in the same conditions is marked with * P< 0.05; ** P< 0.01; *** P< 0.001; ****P< 0.0001 based on ANOVA followed by *post-hoc* testing for multiple comparisons.

587
588

589 In low-organic dry conditions the Ag1_LD and Ag2_LD populations survived better than the ancestor
590 possibly due to carrying mutations in *ompC*, *zraS* and *apt*. The mutations carried by Ag3_LD – Ag5_LD,
591 although some of them in genes involved in stress responses (*argT*), possibly affecting cell surface
592 properties (*wza*, *anmK*, *slyB*) or transport function (*yegH*), do not measurably change metal surface

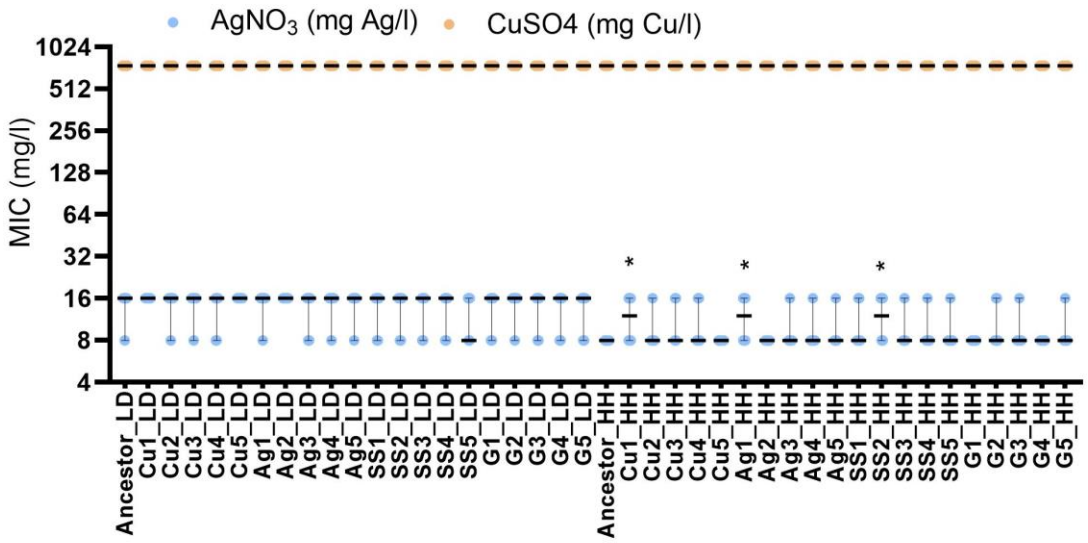
593 tolerance which does not rule out possible benefit, but multiple mutations could be needed to achieve a
594 substantially more tolerant phenotype.

595 Cu1_LD and Cu2_LD populations presented no survival benefit over the ancestor, at least partly due to
596 extremely high variability of viable counts. Significantly better survival of Cu3_LD-C5_LD could be
597 explained by possibly beneficial mutations in *rssB (*and/or *ybiB)*, *yfhM (*and/or IS-mediated deletion of
598 *EcolC2715-EcolC_2745)*, and *cysN* (and/or IS-interrupted *EcolC_3887)*, respectively.

599 **Phenotypic characterization of evolved populations: metal salt and antibiotic tolerance**

600 Release of metal ions is considered to be one of the main biocidal mechanisms of antimicrobial metal
601 surfaces [141], [142]. To evaluate copper and silver ion tolerance of the evolved populations and isolates
602 carrying mutations of interest two approaches were used, agar microdilution assay with copper and
603 silver salts to assess minimal inhibitory concentration (MIC) [44] and a modified minimal biocidal
604 concentration (MBC) assay [45]. Evolutionary changes in growth (assessed as MIC) and survival (assessed
605 by MBC) in the presence of an antimicrobial substance might occur independently of each other and
606 increased survival might be detected in case of unchanged MIC [143]. Furthermore, survival of metal-
607 exposed bacteria is heavily dependent on organic content of exposure media [45], [144] and can cause
608 up to 1000-fold differences in MBC value [45]. Evolution of tolerance can be difficult to interpret due to
609 involvement of multiple bacterial defense mechanisms, affected by genomic and transcriptomic changes
610 or even global stereotypic transcriptome expression patterns [145].

611



612 **Figure 6. Minimal inhibitory concentration (MIC) of copper and silver of the evolved populations and**
613 **their respective ancestors**. Copper and silver salts were used as a source of respective metal ions for MIC
614 assay on Mueller-Hinton agar Values from three biological replicates and two technical replicates are
615 presented with median and range. Statistical significance of the difference from the ancestor exposed in
616 the same conditions is marked with * P< 0.05.

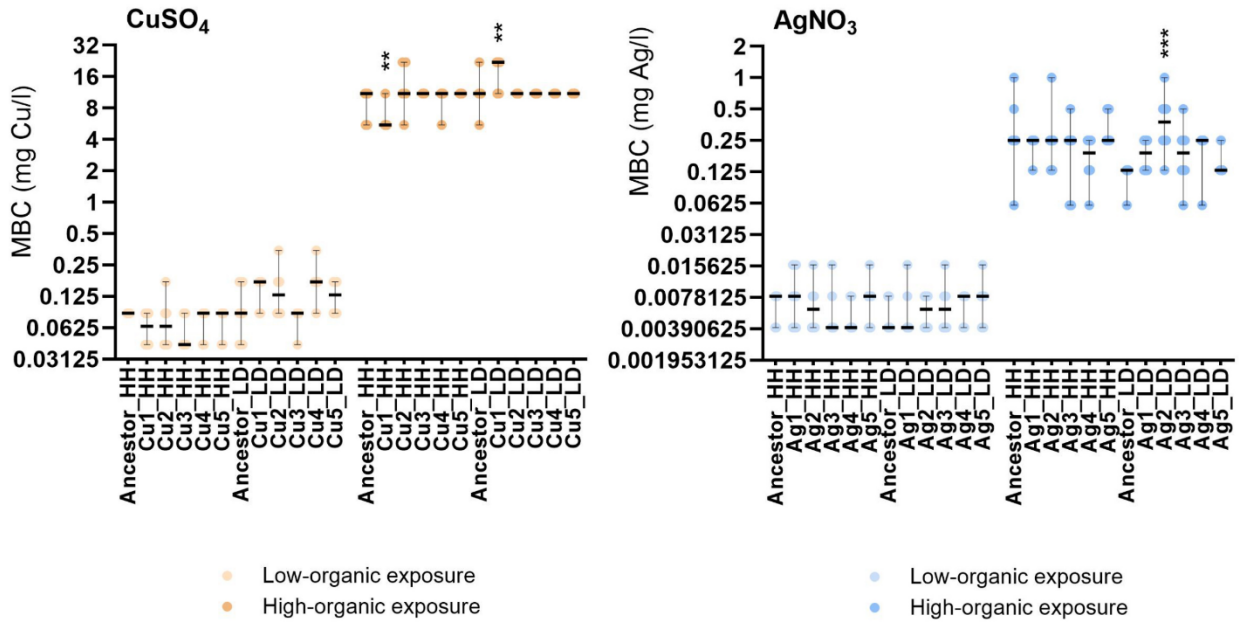


Figure 7. Minimal biocidal concentration (MBC) of copper and silver of the evolved populations and their respective ancestors. Populations evolved on copper surfaces were tested against copper (left panel) and lines evolved on silver against silver (right panel). Values from three independent experiments (6 data points) are presented with median and range. Statistical significance of the difference from the ancestor exposed in the same conditions is marked with ** P< 0.01; *** P<0.001.

No changes in the respective Cu and Ag salt MIC values of the copper- and silver-exposed evolved populations compared to ancestor were observed (Fig 6). Changes in MBC values were small and rather explained by the environmental parameters of the evolution experiment not by the enriched mutations (Fig 7) possibly indicating a non-genetic acclimatization effect. For example, populations evolved on copper in LD conditions tolerated higher Cu ion concentration in MBC assay in water and populations evolved in LD conditions on silver tolerated higher Ag ion concentration in high-organic medium. That could be due to copper but not silver acidifying the high-organic medium causing additional stress as compared to high organic content offering more energy resources to counteract silver toxicity without the additional acid stress. Copper-exposed populations not being more tolerant to ionic copper is in good agreement with Bleichert *et al*. [31] who also found no increase in ionic copper tolerance of the bacteria evolved on copper surfaces despite demonstrating increased copper surface tolerance. Also, copper tolerant bacteria isolated from dry copper alloy surfaces have demonstrated no increase in copper ion tolerance in liquid conditions [146].

Slightly lower Ag MIC (Fig 6) and higher Ag MBC (Fig 7) of the ancestor of the HH evolution experiment compared to ancestor of the LD experiment might arise from mutations in *fliI* and *oppA* present in the HH ancestor (Supplementary table S2) rendering the initial inoculum and resulting populations immotile and possibly interfere with periplasmic oligopeptide sequestering and transport. The mutations had no effect on copper (Fig 6 and 7) nor antibiotic tolerance (Fig 8). This could indicate that differently from copper and tested antibiotics, *E. coli* slightly benefits from either motility or periplasmic oligopeptide sequestering while growing on silver-supplemented agar medium and potential disruption of the same functions increases survival in the presence of higher silver concentrations in liquid environment. One

644 could expect immotility to affect both silver and copper tolerance the same way by being able to move
645 away from the toxicant, especially form solid surfaces releasing high local concentrations of toxic ions
646 closer to the metal surface. Alternatively, energy saved at the expense of motility could be used for
647 active defense mechanisms. On the other hand, silver has higher affinity to proteins then copper,
648 especially at their cysteine residues [147], [148] and in that context the *oppA* mutation could affect
649 silver-bound oligopeptide uptake which makes *oppA* a better causal candidate of the ancestral tolerance
650 difference in MIC and MBC assays of the evolved populations. Interestingly, neither of the mutation in
651 HH ancestor significantly affected survival or growth on silver and copper surfaces (Supplementary
652 Figure S5) highlighting exposure-specific employment of defensive strategies. However, different genetic
653 context of HH and LD ancestors could have affected the evolutionary scenarios observed during the
654 evolution experiment.

655 From post-evolution MIC and MBC tests single colonies with seemingly substantially higher Cu or Ag
656 tolerance were isolated and retested to detect colonies with reproducibly increased metal ion tolerance.
657 Secondary isolates from $AgNO_3$ MBC tests in high-organic exposure medium demonstrating persistent
658 increase in copper and silver ion tolerance carried independent *silS* mutations (full mutational profiles
659 described in Supplementary Table S2) not detected in their population of origin (Ag5_LD and Ag4_HH).
660 Different *silS* point mutations emerging from silver-exposed populations not detectable before the liquid
661 silver challenge in MBC assay indicated rapid selection of *silS* mutants in liquid, but not surface format.

662 Antibiotic tolerance of the evolved populations did not change substantially compared to ancestors and
663 no newly acquired resistances were observed. Detected changes in antibiotic tolerance compared to
664 ancestors were not easily explained by genetic background and were not bigger than 2-fold compared to
665 ancestor (Fig 8).

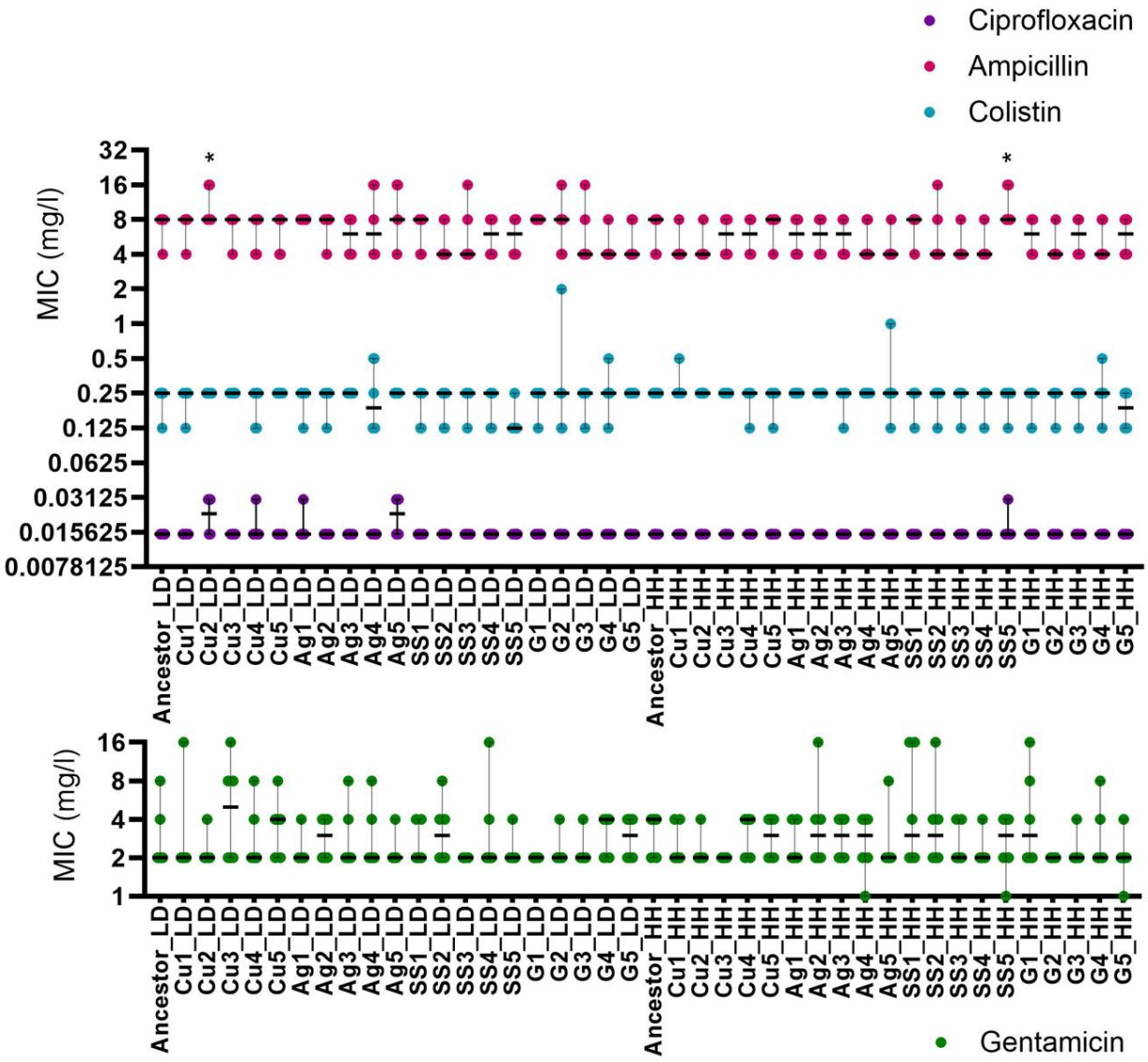


Figure 8. Antibiotic tolerance of the evolved populations and their respective ancestors, minimal inhibitory concentration (MIC). Values of at least 6 data points are presented with median and range. Statistical significance of the difference of a single evolved lineage from the ancestor exposed in the same conditions is marked with * P< 0.05.

Phenotypic characterization of selected mutants

Isolates from evolved populations carrying mutations of interest (*tnaA* M266I, *ydjN*::IS1) associated with sulfur amino acid metabolism, and secondary isolates from metal tolerance assays of the evolved populations with increased copper and silver ion tolerance carrying *silS* point mutations (described in Supplementary Table S2) were characterized separately to reduce the variability generated by

679 heterogeneity in the evolved populations. The secondary isolates carrying *silS* mutations did not
680 demonstrate substantial advantage in surface format (Fig 9). However, *silS* mutants presented higher
681 tolerance of copper and silver exposure and were at least 8 times more resistant to silver (grew at
682 highest tested MIC concentration; Fig. 10) in liquid MBC and MIC tests highlighting the critical
683 importance of exposure conditions not only in antimicrobial surface efficacy testing but also in tolerance
684 mechanisms and risk assessment. Interestingly, out of the tested bacteria, the *silS* mutants were also
685 less sensitive to ampicillin and ciprofloxacin than the wild type (Fig 11). *Sil* mutants have also previously
686 been demonstrated to be less susceptible to ampicillin and ciprofloxacin [35]. Interrupted *ydjN* on the
687 other hand enhanced growth on both copper (Fig 9B) and silver (Fig 9D) surfaces in high-organic humid
688 conditions but also increased survival in liquid silver and copper exposures (Fig 10B). Although reaching
689 higher mean CFU/surface values than wild type, mutant *tnaA* isolate did not demonstrate any
690 statistically significant advantage over wild type in surface tolerance, MBC or MIC tests (Fig 9 and 10).
691 This could be either due to small effect size or the negative effect of an additional IS1-mediated
692 multigene deletion in the *tnaA* M266I isolate (Supplementary Table S2).

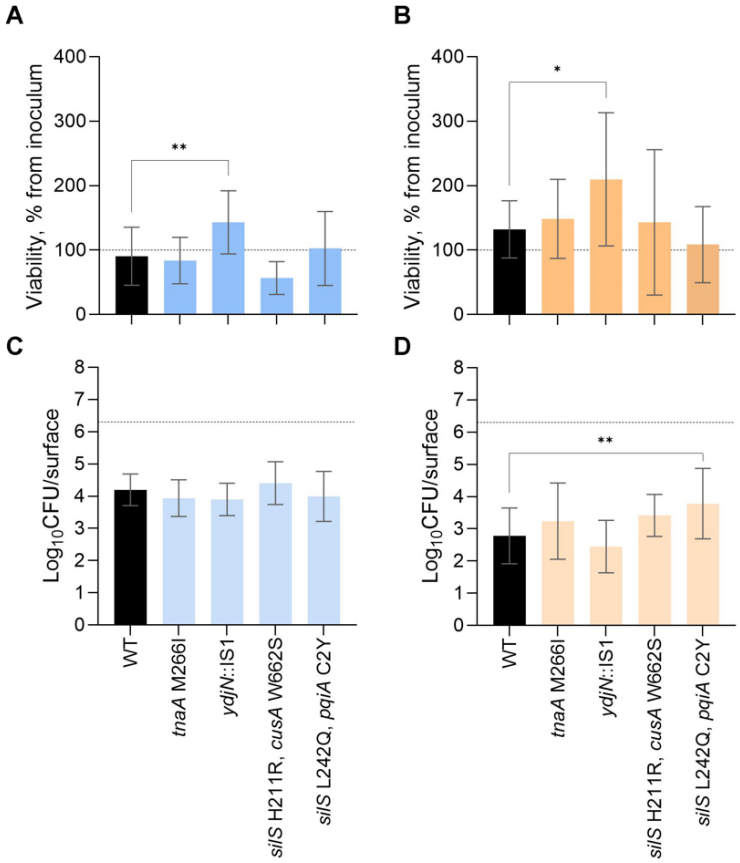


Figure 9. Silver (A,C) and copper (B, D) surface tolerance of the selected mutants and wild-type(WT) in high-organic humid (A,B) and low-organic dry (C,D) and conditions. Values from three independent experiments (6 surface exposures per condition) are presented with mean ±SD. Ancestral viability (A,B) or pre-exposure inoculum baseline (C,D) is marked with a dotted line. Statistical significance of the difference from the ancestor exposed in the same conditions is marked with * P< 0.05; ** P< 0.01 based on non-parametric ANOVA followed by *post-hoc* testing for multiple comparisons.

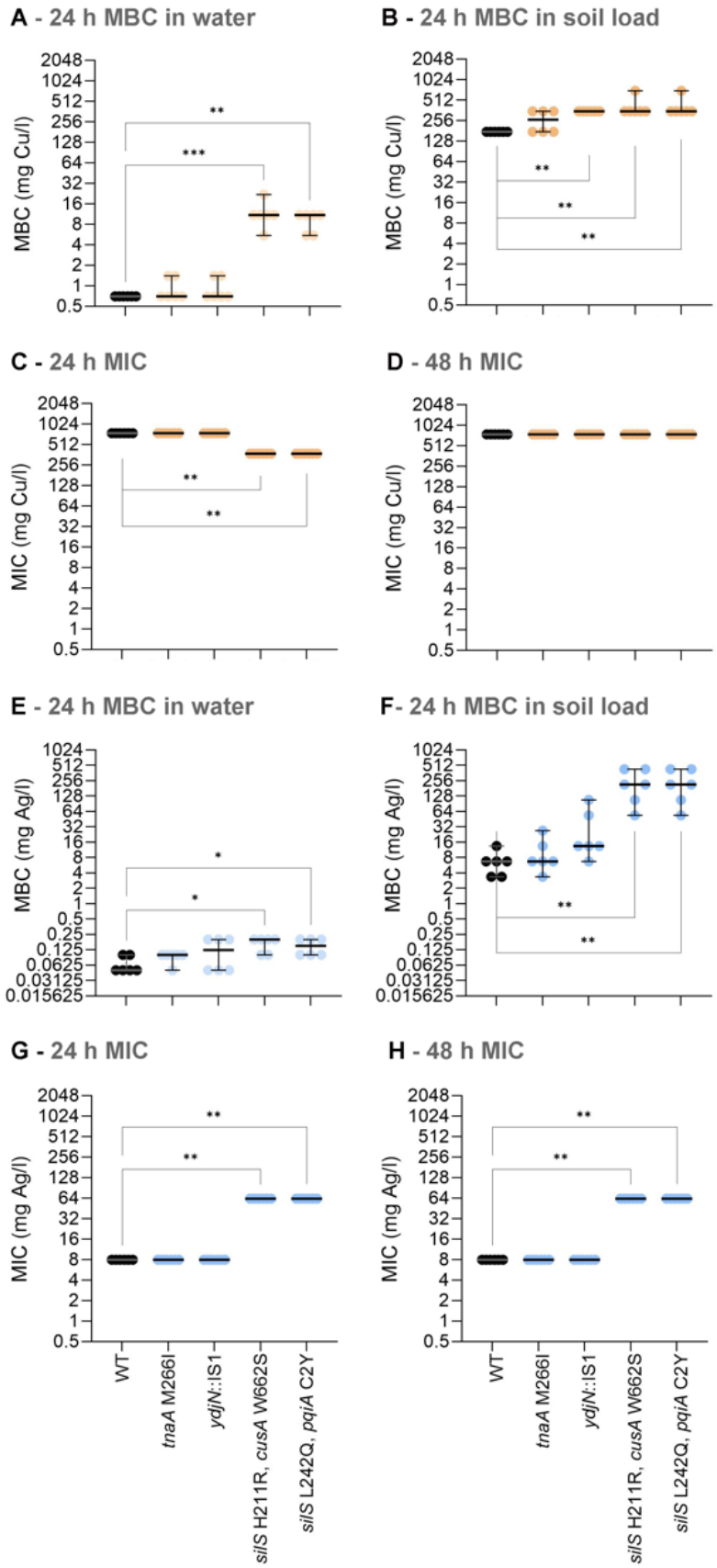


Figure 10. MBC and MIC values of copper (A-D, orange) and silver (E-H, blue) salts of selected mutants and wild-type (WT). MBC in water (A, E) and organic soil load (B, F) and MIC values at 24 h (C, G) and 48 h (D, H) are presented. On panels G and H MIC is ≥ 64 mg Ag/l as the detected value was the highest silver concentration tested in MIC assay. Values from three independent experiments with 6 data points are presented with median and range. Statistical significance of the difference from the ancestor exposed in the same conditions is marked with * P< 0.05; ** P< 0.01; *** P<0.001 based on non-parametric ANOVA followed by *post-hoc* testing for multiple comparisons.

729

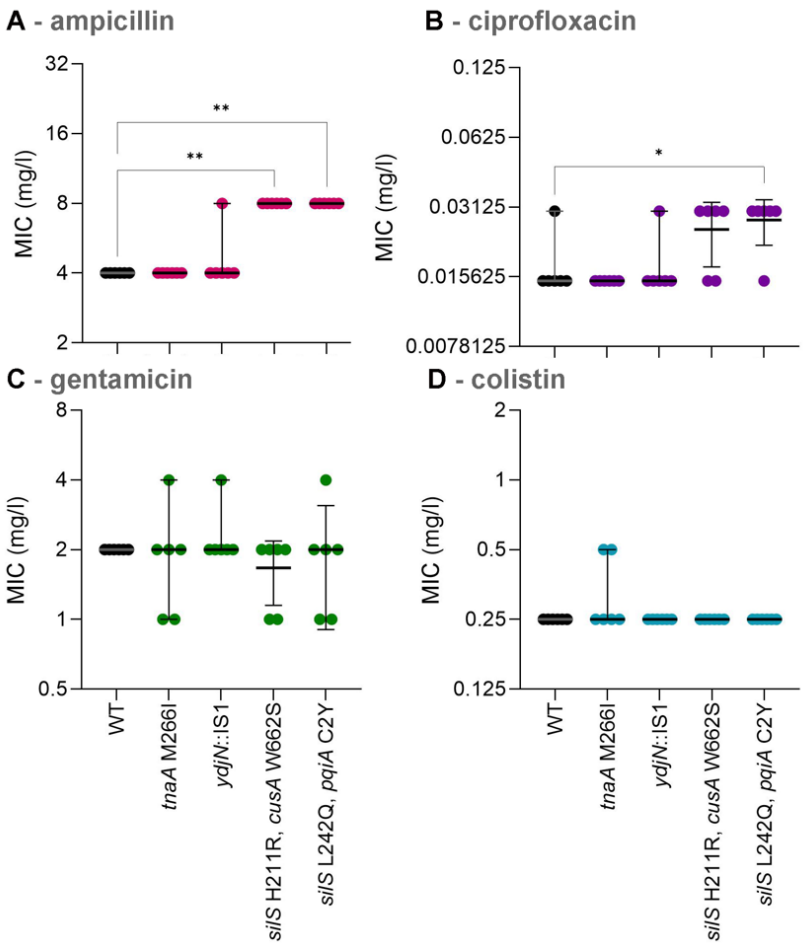


Figure 11. Ampicillin (A), ciprofloxacin (B), gentamicin (C) and colistin (D) tolerance of the selected mutants and wild type (WT). Values from three independent experiments with 6 data points are presented with median and range. Statistical significance of the difference from wild-type exposed in the same conditions is marked with * P< 0.05; ** P< 0.01 based on non-parametric ANOVA followed by *post-hoc* testing for multiple comparisons.

752 **Conclusion**

753 • Repeated exposure of *E. coli* ATCC 8739 to copper and silver surfaces in environmentally
754 exposed conditions did not increase the number of mutations accumulated in the exposed
755 populations indicating reduced risk of pathogenicity-associated hypermutator emergence on
756 antimicrobial copper and silver surfaces compared to steel and glass in application-relevant
757 semidry conditions.
758 • Enriched mutations on copper and silver surfaces indicate a shift toward more energy-passive
759 mechanisms of metal tolerance in surface exposure while active efflux is rapidly selected for in a
760 single round of liquid silver challenge.
761 • Cryptic copper and silver tolerance loci *pco* and *sil* were not activated during cyclic surface
762 exposure but mutations in *silS*, activating the *sil*-encoded copper/silver efflux system was rapidly
763 selected for in the following conventional MBC assay indicating decreased risk of *sil* activation in
764 surface exposure conditions compared to copper and silver salts in liquid medium. The *sil*
765 mutants demonstrated substantial growth and survival benefit in MIC and MBC assays with
766 copper and silver salts, but not a clear beneficial effect in copper or silver surface exposure. This

767 indicates reduced risk of activation of the tolerance loci and possible co-selection of antibiotic
768 resistance in case of extrachromosomal genetic elements carrying such metal tolerance loci.

769 • Mutations in several known metal tolerance (*ompC*, *zraS*, *cysN*) and stress response (*rssB*)
770 associated genes were detected in copper or silver exposed populations compared to steel and
771 glass. The abundance of mutations in sulfur and sulfur amino acid metabolism genes (*tnaA,*
772 *ydjN, cysN*) offering slight survival or growth benefit on copper and silver surfaces was surprising
773 but could be plausibly explained by reduced metal uptake and increased metal detoxification by
774 sulfidation. Co-incidentally the high-organic soiling medium used was exceptionally high in
775 cysteine and mutations in other sulfur metabolism genes (*dcyD* in high-organic and *metK* in both
776 exposure conditions) were also accumulated on control surfaces. Therefore, we propose that
777 the benefit of the sulfur metabolism associated mutation in silver and copper exposed
778 populations was probably dependent on both the exposure medium and specific environmental
779 stresses as the benefit was generally not reproduced in liquid exposure.

780 • Several IS1-mediated multigene deletions of variable length were detected irrespective of
781 surface type with clear deletional hotspots possibly indicating strain-specific genomic instability
782 rather than selection of the accumulated deletions. The role of such deletions as well as strain
783 specificity remains to be studied.

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786 **Availability of data**

787 Sequencing data is made available in European Nucleotide Archive (ENA), accession PRJEB75034. Raw
788 numerical data is included figure by figure in Supplementary Data file. Any other inquiries can be
789 directed to the corresponding author.

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793 **Author contribution**

794 MR and SA, experimental design. SA, SU and MR, lab experiments and analysis of the results. SA, MR and
795 AI, interpretation of the results. MR writing of the manuscript. MR and AI editing of the manuscript. AI
796 project management and funding acquisition.

797 **References**

798 [1] M. Rosenberg *et al.*, "Potential ecotoxicological effects of antimicrobial surface coatings: a
799 literature survey backed up by analysis of market reports," *PeerJ*, vol. 7, p. e6315, Feb. 2019, doi:
800 10.7717/peerj.6315.

801 [2] H. Kaur, M. Rosenberg, M. Kook, D. Danilian, V. Kisand, and A. Ivask, "Antibacterial activity of solid
802 surfaces is critically dependent on relative humidity, inoculum volume and organic soiling," *FEMS*
803 *Microbes*, p. xtad022, Dec. 2023, doi: 10.1093/femsmc/xtad022.

804 [3] L. M. Murray, A. Hayes, J. Snape, B. Kasprzyk-Hordern, W. H. Gaze, and A. K. Murray, "Co-selection
805 for antibiotic resistance by environmental contaminants," *npj Antimicrob Resist*, vol. 2, no. 1, p. 9,
806 Apr. 2024, doi: 10.1038/s44259-024-00026-7.

807 [4] P. Vats, U. J. Kaur, and P. Rishi, “Heavy metal-induced selection and proliferation of antibiotic
808 resistance: A review,” *Journal of Applied Microbiology*, vol. 132, no. 6, pp. 4058–4076, Jun. 2022,
809 doi: 10.1111/jam.15492.
810 [5] X. Z. Li, H. Nikaido, and K. E. Williams, “Silver-resistant mutants of Escherichia coli display active
811 efflux of Ag+ and are deficient in porins,” *J Bacteriol*, vol. 179, no. 19, pp. 6127–6132, Oct. 1997,
812 doi: 10.1128/jb.179.19.6127-6132.1997.
813 [6] K. Nishino, E. Nikaido, and A. Yamaguchi, “Regulation of Multidrug Efflux Systems Involved in
814 Multidrug and Metal Resistance of *Salmonella enterica* Serovar Typhimurium,” *J Bacteriol*, vol. 189,
815 no. 24, pp. 9066–9075, Dec. 2007, doi: 10.1128/JB.01045-07.
816 [7] P. Teixeira, M. Tacão, A. Alves, and I. Henriques, “Antibiotic and metal resistance in a ST395
817 Pseudomonas aeruginosa environmental isolate: A genomics approach,” *Marine Pollution Bulletin*,
818 vol. 110, no. 1, pp. 75–81, Sep. 2016, doi: 10.1016/j.marpolbul.2016.06.086.
819 [8] E. Denamur and I. Matic, “Evolution of mutation rates in bacteria,” *Mol Microbiol*, vol. 60, no. 4,
820 pp. 820–827, May 2006, doi: 10.1111/j.1365-2958.2006.05150.x.
821 [9] R. Maddamsetti and N. A. Grant, “Divergent Evolution of Mutation Rates and Biases in the Long-
822 Term Evolution Experiment with Escherichia coli,” *Genome Biology and Evolution*, vol. 12, no. 9,
823 pp. 1591–1603, Sep. 2020, doi: 10.1093/gbe/evaa178.
824 [10] O. Tenaillon, B. Toupance, H. Le Nagard, F. Taddei, and B. Godelle, “Mutators, Population Size,
825 Adaptive Landscape and the Adaptation of Asexual Populations of Bacteria,” *Genetics*, vol. 152, no.
826 2, pp. 485–493, Jun. 1999, doi: 10.1093/genetics/152.2.485.
827 [11] B. H. Good, M. J. McDonald, J. E. Barrick, R. E. Lenski, and M. M. Desai, “The dynamics of molecular
828 evolution over 60,000 generations,” *Nature*, vol. 551, no. 7678, pp. 45–50, Nov. 2017, doi:
829 10.1038/nature24287.
830 [12] A. Couce, N. Alonso-Rodriguez, C. Costas, A. Oliver, and J. Blázquez, “Intrapopulation variability in
831 mutator prevalence among urinary tract infection isolates of Escherichia coli,” *Clinical*
832 *Microbiology and Infection*, vol. 22, no. 6, p. 566.e1-566.e7, Jun. 2016, doi:
833 10.1016/j.cmi.2016.03.008.
834 [13] E. Denamur *et al.*, “High Frequency of Mutator Strains among Human Uropathogenic *Escherichia*
835 *coli* Isolates,” *J Bacteriol*, vol. 184, no. 2, pp. 605–609, Jan. 2002, doi: 10.1128/JB.184.2.605-
836 609.2002.
837 [14] F. Labat, O. Pradillon, L. Garry, M. Peuchmaur, B. Fantin, and E. Denamur, “Mutator phenotype
838 confers advantage in *Escherichia coli* chronic urinary tract infection pathogenesis,” *FEMS*
839 *Immunology & Medical Microbiology*, vol. 44, no. 3, pp. 317–321, Jun. 2005, doi:
840 10.1016/j.femsim.2005.01.003.
841 [15] J. E. LeClerc, B. Li, W. L. Payne, and T. A. Cebula, “High Mutation Frequencies Among *Escherichia*
842 *coli* and *Salmonella* Pathogens,” *Science*, vol. 274, no. 5290, pp. 1208–1211, Nov. 1996, doi:
843 10.1126/science.274.5290.1208.
844 [16] M. Levert *et al.*, “Molecular and Evolutionary Bases of Within-Patient Genotypic and Phenotypic
845 Diversity in Escherichia coli Extraintestinal Infections,” *PLoS Pathog*, vol. 6, no. 9, p. e1001125, Sep.
846 2010, doi: 10.1371/journal.ppat.1001125.
847 [17] V. E. Rees *et al.*, “Characterization of Hypermutator Pseudomonas aeruginosa Isolates from
848 Patients with Cystic Fibrosis in Australia,” *Antimicrob Agents Chemother*, vol. 63, no. 4, pp. e02538-
849 18, Apr. 2019, doi: 10.1128/AAC.02538-18.
850 [18] C. A. Colque *et al.*, “Hypermutator Pseudomonas aeruginosa Exploits Multiple Genetic Pathways To
851 Develop Multidrug Resistance during Long-Term Infections in the Airways of Cystic Fibrosis
852 Patients,” *Antimicrob Agents Chemother*, vol. 64, no. 5, pp. e02142-19, Apr. 2020, doi:
853 10.1128/AAC.02142-19.

854 [19] G. M. Eliopoulos and J. Blazquez, “Hypermutation as a Factor Contributing to the Acquisition of
855 Antimicrobial Resistance,” *Clinical Infectious Diseases*, vol. 37, no. 9, pp. 1201–1209, Nov. 2003,
856 doi: 10.1086/378810.
857 [20] P. P. Khil *et al.*, “Dynamic Emergence of Mismatch Repair Deficiency Facilitates Rapid Evolution of
858 Ceftazidime-Avibactam Resistance in Pseudomonas aeruginosa Acute Infection,” *mBio*, vol. 10, no.
859 5, pp. e01822-19, Oct. 2019, doi: 10.1128/mBio.01822-19.
860 [21] H. H. Mehta *et al.*, “The Essential Role of Hypermutation in Rapid Adaptation to Antibiotic Stress,”
861 *Antimicrob Agents Chemother*, vol. 63, no. 7, pp. e00744-19, Jul. 2019, doi: 10.1128/AAC.00744-
862 19.
863 [22] S. B. I. Schmidt, A. Rodríguez-Rojas, J. Rolff, and F. Schreiber, “Biocides used as material
864 preservatives modify rates of de novo mutation and horizontal gene transfer in bacteria,” *Journal*
865 *of Hazardous Materials*, vol. 437, p. 129280, Sep. 2022, doi: 10.1016/j.jhazmat.2022.129280.
866 [23] H. Shen *et al.*, “Hormetic dose-responses for silver antibacterial compounds, quorum sensing
867 inhibitors, and their binary mixtures on bacterial resistance of Escherichia coli,” *Science of The*
868 *Total Environment*, vol. 786, p. 147464, Sep. 2021, doi: 10.1016/j.scitotenv.2021.147464.
869 [24] X. Li, A. Z. Gu, Y. Zhang, B. Xie, D. Li, and J. Chen, “Sub-lethal concentrations of heavy metals induce
870 antibiotic resistance via mutagenesis,” *Journal of Hazardous Materials*, vol. 369, pp. 9–16, May
871 2019, doi: 10.1016/j.jhazmat.2019.02.006.
872 [25] J. K. Crane, M. B. Cheema, M. A. Olyer, and M. D. Sutton, “Zinc Blockade of SOS Response Inhibits
873 Horizontal Transfer of Antibiotic Resistance Genes in Enteric Bacteria,” *Front. Cell. Infect.*
874 *Microbiol.*, vol. 8, p. 410, Nov. 2018, doi: 10.3389/fcimb.2018.00410.
875 [26] M. L. Buberg, I. L. Witsø, T. M. L’Abée-Lund, and Y. Wasteson, “Zinc and Copper Reduce
876 Conjugative Transfer of Resistance Plasmids from Extended-Spectrum Beta-Lactamase-Producing
877 *Escherichia coli*,” *Microbial Drug Resistance*, vol. 26, no. 7, pp. 842–849, Jul. 2020, doi:
878 10.1089/mdr.2019.0388.
879 [27] E.-M. Saliu, M. Eitinger, J. Zentek, and W. Vahjen, “Nutrition Related Stress Factors Reduce the
880 Transfer of Extended-Spectrum Beta-Lactamase Resistance Genes between an Escherichia coli
881 Donor and a Salmonella Typhimurium Recipient In Vitro,” *Biomolecules*, vol. 9, no. 8, p. 324, Jul.
882 2019, doi: 10.3390/biom9080324.
883 [28] M. Rosenberg, H. Vija, A. Kahru, C. W. Keevil, and A. Ivask, “Rapid in situ assessment of Cu-ion
884 mediated effects and antibacterial efficacy of copper surfaces,” *Scientific Reports*, vol. 8, no. 1,
885 Dec. 2018, doi: 10.1038/s41598-018-26391-8.
886 [29] S. Liu and X.-X. Zhang, “Small colony variants are more susceptible to copper-mediated contact
887 killing for Pseudomonas aeruginosa and Staphylococcus aureus,” *Journal of Medical Microbiology*,
888 vol. 65, no. 10, pp. 1143–1151, Oct. 2016, doi: 10.1099/jmm.0.000348.
889 [30] C. E. Santo *et al.*, “Bacterial Killing by Dry Metallic Copper Surfaces,” *Appl. Environ. Microbiol.*, vol.
890 77, no. 3, pp. 794–802, Feb. 2011, doi: 10.1128/AEM.01599-10.
891 [31] P. Bleichert *et al.*, “Mutant Strains of Escherichia coli and Methicillin-Resistant Staphylococcus
892 aureus Obtained by Laboratory Selection To Survive on Metallic Copper Surfaces,” *Appl Environ*
893 *Microbiol*, vol. 87, no. 1, pp. e01788-20, Dec. 2020, doi: 10.1128/AEM.01788-20.
894 [32] F. Xu, S. Liu, N. Naren, L. Li, L. Z. Ma, and X. Zhang, “Experimental evolution of bacterial survival on
895 metallic copper,” *Ecology and Evolution*, vol. 12, no. 8, Aug. 2022, doi: 10.1002/ece3.9225.
896 [33] H. Wang *et al.*, “Characterization of Silver Resistance and Coexistence of sil Operon with Antibiotic
897 Resistance Genes Among Gram-Negative Pathogens Isolated from Wound Samples by Using
898 Whole-Genome Sequencing,” *IDR*, vol. Volume 15, pp. 1425–1437, Mar. 2022, doi:
899 10.2147/IDR.S358730.

[34] B. M. Staehlin, J. G. Gibbons, A. Rokas, T. V. O'Halloran, and J. C. Slot, "Evolution of a heavy metal homeostasis/resistance island reflects increasing copper stress in Enterobacteria," *Genome Biol Evol*, p. evw031, Feb. 2016, doi: 10.1093/gbe/evw031.

[35] M. Blanco Massani *et al.*, "Chromosomal Sil system contributes to silver resistance in E. coli ATCC 8739," *Biometals*, vol. 31, no. 6, pp. 1101–1114, Dec. 2018, doi: 10.1007/s10534-018-0143-1.

[36] C. A. Woolley, J. M. Sutton, and M. E. Wand, "Mutations in SilS and CusS/OmpC represent different routes to achieve high level silver ion tolerance in Klebsiella pneumoniae," *BMC Microbiol*, vol. 22, no. 1, p. 113, Dec. 2022, doi: 10.1186/s12866-022-02532-y.

[37] M. Ashburner *et al.*, "Gene Ontology: tool for the unification of biology," *Nat Genet*, vol. 25, no. 1, pp. 25–29, May 2000, doi: 10.1038/75556.

[38] D. Szklarczyk *et al.*, "The STRING database in 2023: protein–protein association networks and functional enrichment analyses for any sequenced genome of interest," *Nucleic Acids Research*, vol. 51, no. D1, pp. D638–D646, Jan. 2023, doi: 10.1093/nar/gkac1000.

[39] CEN, "EN 13697:2015+A1:2019 Chemical disinfectants and antiseptics - Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas - Test method and requirements without mechanical action (phase 2, step 2)." 2015.

[40] EPA, "Test Method for Evaluating the Efficacy of Antimicrobial Surface Coatings MB-40-00." Sep. 26, 2022. Accessed: Jan. 18, 2022. [Online]. Available: https://www.epa.gov/pesticide-analytical-methods/antimicrobial-testing-methods-procedures-mb-40-00

[41] ISO, "ISO 22196:2011 Measurement of antibacterial activity on plastics and other non-porous surfaces." 2011.

[42] EUCAST, "EUCAST definitions of clinical breakpoints and epidemiological cut-off values." EUCAST. Accessed: May 30, 2024. [Online]. Available: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/EUCAST_SOPs/EUCAST_definitions_of_clinical_breakpoints_and_ECOFFs.pdf

[43] G. Kahlmeter and J. Turnidge, "How to: ECOFFs—the why, the how, and the don'ts of EUCAST epidemiological cutoff values," *Clinical Microbiology and Infection*, vol. 28, no. 7, pp. 952–954, Jul. 2022, doi: 10.1016/j.cmi.2022.02.024.

[44] EUCAST, "Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution," *Clinical Microbiology and Infection*, vol. 6, no. 9, pp. 509–515, Sep. 2000, doi: 10.1046/j.1469-0691.2000.00142.x.

[45] S. Suppi *et al.*, "A novel method for comparison of biocidal properties of nanomaterials to bacteria, yeasts and algae," *Journal of Hazardous Materials*, vol. 286, pp. 75–84, Apr. 2015, doi: 10.1016/j.jhazmat.2014.12.027.

[46] D. E. Deatherage and J. E. Barrick, "Identification of Mutations in Laboratory-Evolved Microbes from Next-Generation Sequencing Data Using breseq," in *Engineering and Analyzing Multicellular Systems*, vol. 1151, L. Sun and W. Shou, Eds., in Methods in Molecular Biology, vol. 1151. , New York, NY: Springer New York, 2014, pp. 165–188. doi: 10.1007/978-1-4939-0554-6_12.

[47] J. E. Barrick *et al.*, "Identifying structural variation in haploid microbial genomes from short-read resequencing data using breseq," *BMC Genomics*, vol. 15, no. 1, p. 1039, 2014, doi: 10.1186/1471-2164-15-1039.

[48] P. Siguier, "ISfinder: the reference centre for bacterial insertion sequences," *Nucleic Acids Research*, vol. 34, no. 90001, pp. D32–D36, Jan. 2006, doi: 10.1093/nar/gkj014.

[49] H. Lee, E. Popodi, H. Tang, and P. L. Foster, "Rate and molecular spectrum of spontaneous mutations in the bacterium *Escherichia coli* as determined by whole-genome sequencing," *Proc. Natl. Acad. Sci. U.S.A.*, vol. 109, no. 41, Oct. 2012, doi: 10.1073/pnas.1210309109.

[50] R. P. Maharjan, T. Ferenci, P. R. Reeves, Y. Li, B. Liu, and L. Wang, “The multiplicity of divergence mechanisms in a single evolving population,” *Genome Biol*, vol. 13, no. 6, p. R41, 2012, doi: 10.1186/gb-2012-13-6-r41.

[51] Z. Zou and J. Zhang, “Are Nonsynonymous Transversions Generally More Deleterious than Nonsynonymous Transitions?,” *Molecular Biology and Evolution*, vol. 38, no. 1, pp. 181–191, Jan. 2021, doi: 10.1093/molbev/msaa200.

[52] R. E. Hudson, “Transcription increases multiple spontaneous point mutations in Salmonella enterica,” *Nucleic Acids Research*, vol. 31, no. 15, pp. 4517–4522, Aug. 2003, doi: 10.1093/nar/gkg651.

[53] I. J. Fijalkowska, R. L. Dunn, and R. M. Schaaper, “Genetic requirements and mutational specificity of the Escherichia coli SOS mutator activity,” *J Bacteriol*, vol. 179, no. 23, pp. 7435–7445, Dec. 1997, doi: 10.1128/jb.179.23.7435-7445.1997.

[54] A. Sakai, M. Nakanishi, K. Yoshiyama, and H. Maki, “Impact of reactive oxygen species on spontaneous mutagenesis in *Escherichia coli*,” *Genes to Cells*, vol. 11, no. 7, pp. 767–778, Jul. 2006, doi: 10.1111/j.1365-2443.2006.00982.x.

[55] J. Ruiz-Laguna, “Hydrogen peroxide and coffee induce G:C->T:A transversions in the lacI gene of catalase-defective Escherichia coli,” *Mutagenesis*, vol. 14, no. 1, pp. 95–102, Jan. 1999, doi: 10.1093/mutage/14.1.95.

[56] V. Hodgkinson and M. J. Petris, “Copper Homeostasis at the Host-Pathogen Interface,” *Journal of Biological Chemistry*, vol. 287, no. 17, pp. 13549–13555, Apr. 2012, doi: 10.1074/jbc.R111.316406.

[57] B. Py and F. Barras, “Building Fe–S proteins: bacterial strategies,” *Nat Rev Microbiol*, vol. 8, no. 6, pp. 436–446, Jun. 2010, doi: 10.1038/nrmicro2356.

[58] C. T. Archer *et al.*, “The genome sequence of E. coli W (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of E. coli,” *BMC Genomics*, vol. 12, no. 1, p. 9, Dec. 2011, doi: 10.1186/1471-2164-12-9.

[59] C. Turlan and M. Chandler, “IS1-mediated intramolecular rearrangements: formation of excised transposon circles and replicative deletions.,” *The EMBO Journal*, vol. 14, no. 21, pp. 5410–5421, Nov. 1995, doi: 10.1002/j.1460-2075.1995.tb00225.x.

[60] G. R. Plague *et al.*, “Transposable Elements Mediate Adaptive Debilitation of Flagella in Experimental Escherichia coli Populations,” *J Mol Evol*, vol. 84, no. 5–6, pp. 279–284, Jun. 2017, doi: 10.1007/s00239-017-9797-5.

[61] Y. Wan *et al.*, “IS *1* -related large-scale deletion of chromosomal regions harbouring oxygen-insensitive nitroreductase gene *nfsB* causes nitrofurantoin heteroresistance in *Escherichia coli*,” Microbiology, preprint, Apr. 2023. doi: 10.1101/2023.04.03.535367.

[62] P. A. Calvo, V. Mateo-Cáceres, S. Díaz-Arco, M. Redrejo-Rodríguez, and M. de Vega, “The enterohemorrhagic *Escherichia coli* insertion sequence-excision enhancer protein is a DNA polymerase with microhomology-mediated end-joining activity,” *Nucleic Acids Research*, vol. 51, no. 3, pp. 1189–1207, Feb. 2023, doi: 10.1093/nar/gkad017.

[63] R. Kishino, T. Saito, S. Muto, Y. Tomita, and Y. Sekine, “Insertion sequence excision is enhanced by a protein that catalyzes branch migration and promotes microhomology-mediated end joining,” *Genes to Cells*, vol. 29, no. 2, pp. 131–149, Feb. 2024, doi: 10.1111/gtc.13090.

[64] K. Nakamura, K. Seto, J. Isobe, I. Taniguchi, Y. Gotoh, and T. Hayashi, “Insertion Sequence (IS)-Excision Enhancer (IEE)-Mediated IS Excision from the *lacZ* Gene Restores the Lactose Utilization Defect of Shiga Toxin-Producing Escherichia coli O121:H19 Strains and Is Responsible for Their Delayed Lactose Utilization Phenotype,” *Appl Environ Microbiol*, vol. 88, no. 16, pp. e00760-22, Aug. 2022, doi: 10.1128/aem.00760-22.

[65] M. Razavi, E. Kristiansson, C.-F. Flach, and D. G. J. Larsson, “The Association between Insertion Sequences and Antibiotic Resistance Genes,” *mSphere*, vol. 5, no. 5, pp. e00418-20, Oct. 2020, doi: 10.1128/mSphere.00418-20.

[66] K. Yamanaka, W. Zheng, E. Crooke, Y. Wang, and M. Inouye, “CspD, a novel DNA replication inhibitor induced during the stationary phase in *Escherichia coli*,” *Molecular Microbiology*, vol. 39, no. 6, pp. 1572–1584, Mar. 2001, doi: 10.1046/j.1365-2958.2001.02345.x.

[67] Y. Kim *et al.*, “*Escherichia coli* toxin/antitoxin pair MqsR/MqsA regulate toxin CspD,” *Environmental Microbiology*, vol. 12, no. 5, pp. 1105–1121, May 2010, doi: 10.1111/j.1462-2920.2009.02147.x.

[68] Y. Kim and T. K. Wood, “Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in Escherichia coli,” *Biochemical and Biophysical Research Communications*, vol. 391, no. 1, pp. 209–213, Jan. 2010, doi: 10.1016/j.bbrc.2009.11.033.

[69] D. Binns, E. Dimmer, R. Huntley, D. Barrell, C. O’Donovan, and R. Apweiler, “QuickGO: a web-based tool for Gene Ontology searching,” *Bioinformatics*, vol. 25, no. 22, pp. 3045–3046, Nov. 2009, doi: 10.1093/bioinformatics/btp536.

[70] P. Li *et al.*, “PCOB is a defense outer membrane protein that facilitates cellular uptake of copper,” *Protein Science*, vol. 31, no. 7, p. e4364, Jul. 2022, doi: 10.1002/pro.4364.

[71] K. Bondarczuk and Z. Piotrowska-Seget, “Molecular basis of active copper resistance mechanisms in Gram-negative bacteria,” *Cell Biol Toxicol*, vol. 29, no. 6, pp. 397–405, Dec. 2013, doi: 10.1007/s10565-013-9262-1.

[72] T. J. Tetaz and R. K. Luke, “Plasmid-controlled resistance to copper in Escherichia coli,” *J Bacteriol*, vol. 154, no. 3, pp. 1263–1268, Jun. 1983, doi: 10.1128/jb.154.3.1263-1268.1983.

[73] C. P. Randall, A. Gupta, N. Jackson, D. Busse, and A. J. O’Neill, “Silver resistance in Gram-negative bacteria: a dissection of endogenous and exogenous mechanisms,” *Journal of Antimicrobial Chemotherapy*, vol. 70, no. 4, pp. 1037–1046, Apr. 2015, doi: 10.1093/jac/dku523.

[74] C. Arrault *et al.*, “The battle for silver binding: How the interplay between the SilE, SilF, and SilB proteins contributes to the silver efflux pump mechanism,” *Journal of Biological Chemistry*, vol. 299, no. 8, p. 105004, Aug. 2023, doi: 10.1016/j.jbc.2023.105004.

[75] S. P. T. Hooton *et al.*, “Laboratory Stock Variants of the Archetype Silver Resistance Plasmid pMG101 Demonstrate Plasmid Fusion, Loss of Transmissibility, and Transposition of Tn7/pco/sil Into the Host Chromosome,” *Front. Microbiol.*, vol. 12, p. 723322, Aug. 2021, doi: 10.3389/fmicb.2021.723322.

[76] A. Gupta, K. Matsui, J.-F. Lo, and S. Silver, “Molecular basis for resistance to silver cations in Salmonella,” *Nat Med*, vol. 5, no. 2, pp. 183–188, Feb. 1999, doi: 10.1038/5545.

[77] R. M. Lithgo *et al.*, “The adaptability of the ion-binding site by the Ag(I)/Cu(I) periplasmic chaperone SilF,” *Journal of Biological Chemistry*, vol. 299, no. 11, p. 105331, Nov. 2023, doi: 10.1016/j.jbc.2023.105331.

[78] T. Paysan-Lafosse *et al.*, “InterPro in 2022,” *Nucleic Acids Research*, vol. 51, no. D1, pp. D418–D427, Jan. 2023, doi: 10.1093/nar/gkac993.

[79] R. Taher and E. De Rosny, “A structure-function study of ZraP and ZraS provides new insights into the two-component system Zra,” *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1865, no. 3, p. 129810, Mar. 2021, doi: 10.1016/j.bbagen.2020.129810.

[80] I. Petit-Härtlein *et al.*, “Biophysical and physiological characterization of ZraP from *Escherichia coli* , the periplasmic accessory protein of the atypical ZraSR two-component system,” *Biochemical Journal*, vol. 472, no. 2, pp. 205–216, Dec. 2015, doi: 10.1042/BJ20150827.

[81] K. Rome *et al.*, “The Two-Component System ZraPSR Is a Novel ESR that Contributes to Intrinsic Antibiotic Tolerance in Escherichia coli,” *Journal of Molecular Biology*, vol. 430, no. 24, pp. 4971–4985, Dec. 2018, doi: 10.1016/j.jmb.2018.10.021.

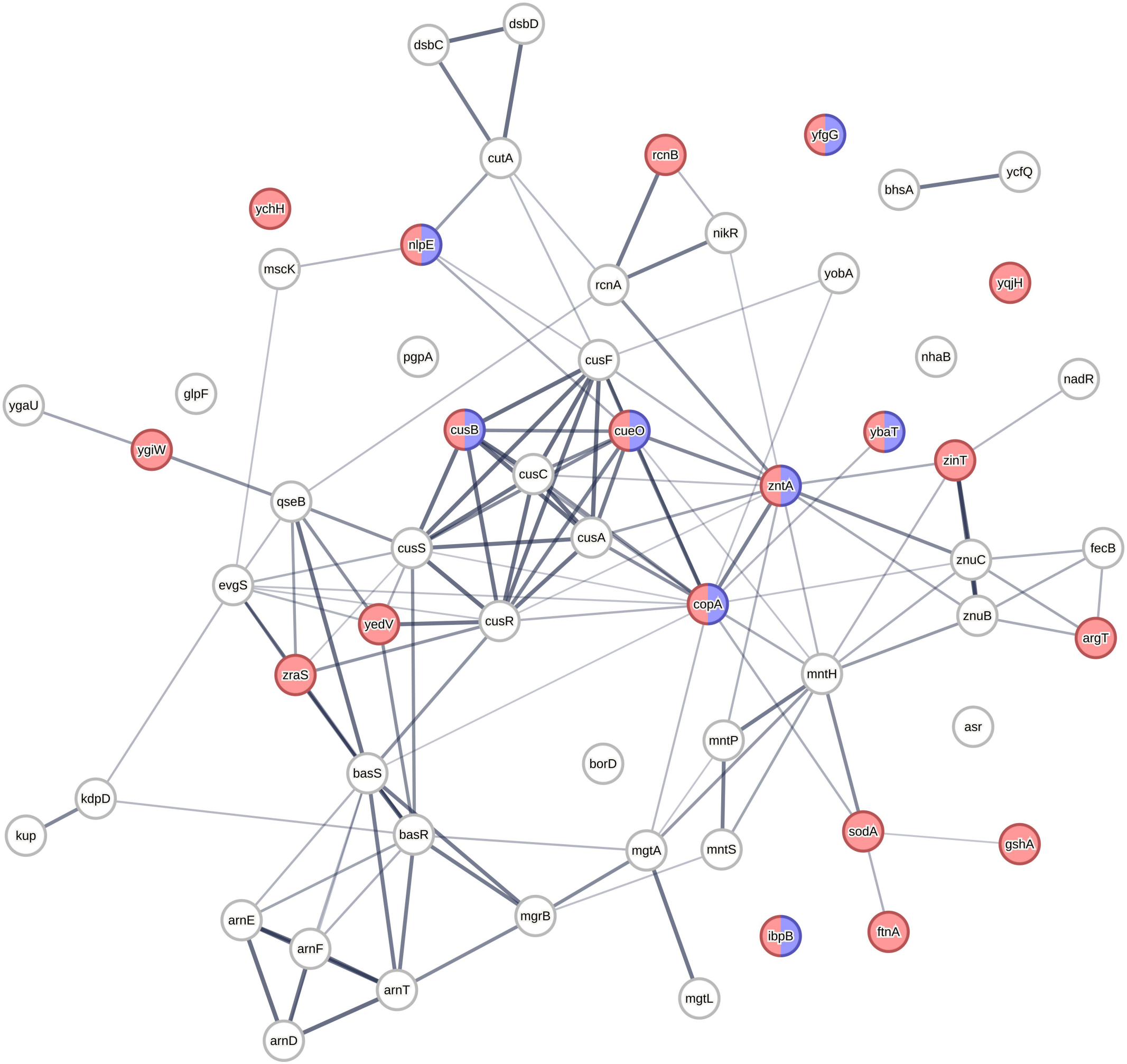
1040 [82] S. Bury-Moné *et al.*, “Global Analysis of Extracytoplasmic Stress Signaling in Escherichia coli,” *PLoS*
1041 *Genet*, vol. 5, no. 9, p. e1000651, Sep. 2009, doi: 10.1371/journal.pgen.1000651.
1042 [83] D. M. Macritchie and T. L. Raivio, “Envelope Stress Responses,” *EcoSal Plus*, vol. 3, no. 2, p.
1043 10.1128/ecosalplus.5.4.7, Dec. 2009, doi: 10.1128/ecosalplus.5.4.7.
1044 [84] M. A. Radzig, V. A. Nadtochenko, O. A. Koksharova, J. Kiwi, V. A. Lipasova, and I. A. Khmel,
1045 “Antibacterial effects of silver nanoparticles on gram-negative bacteria: Influence on the growth
1046 and biofilms formation, mechanisms of action,” *Colloids and Surfaces B: Biointerfaces*, vol. 102, pp.
1047 300–306, Feb. 2013, doi: 10.1016/j.colsurfb.2012.07.039.
1048 [85] X. Chen *et al.*, “Mosaic Evolution of Beta-Barrel-Porin-Encoding Genes in *Escherichia coli*,” *Appl*
1049 *Environ Microbiol*, vol. 88, no. 7, pp. e00060-22, Apr. 2022, doi: 10.1128/aem.00060-22.
1050 [86] Copeland A, Lucas S, Lapidus A, Glavina Del Rio T, Dalin E, Tice H, Bruce D, Goodwin L, Pitluck S,
1051 Kiss H, Brettin T, Detter C, Han JC, Kuske CR, Schmutz J, Larimer F, Land M, Hauser L, Kyrpides N,
1052 Mikhailova N, Ingra P, “Escherichia coli ATCC 8739. A strain of E. coli lacking the major porin
1053 OmpC.” 2008. [Online]. Available: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA18083
1054 [87] D. Schneider, E. Duperchy, J. Depeyrot, E. Coursange, R. E. Lenski, and M. Blot, “Genomic
1055 comparisons among Escherichia coli strains B, K-12, and O157:H7 using IS elements as molecular
1056 markers,” *BMC Microbiol*, vol. 2, no. 1, p. 18, 2002, doi: 10.1186/1471-2180-2-18.
1057 [88] A. Schumann, A. R. Cohn, A. Gaballa, and M. Wiedmann, “Escherichia coli B-Strains Are Intrinsically
1058 Resistant to Colistin and Not Suitable for Characterization and Identification of *mcr* Genes,”
1059 *Microbiol Spectr*, vol. 11, no. 3, pp. e00894-23, Jun. 2023, doi: 10.1128/spectrum.00894-23.
1060 [89] N. Doan and P. G. W. Gettins, “α-Macroglobulins Are Present in Some Gram-negative Bacteria,”
1061 *Journal of Biological Chemistry*, vol. 283, no. 42, pp. 28747–28756, Oct. 2008, doi:
1062 10.1074/jbc.M803127200.
1063 [90] A. Budd, S. Blandin, E. A. Levashina, and T. J. Gibson, “Bacterial α2-macroglobulins: colonization
1064 factors acquired by horizontal gene transfer from the metazoan genome?,” *Genome Biol*, vol. 5,
1065 no. 6, p. R38, May 2004, doi: 10.1186/gb-2004-5-6-r38.
1066 [91] A. Giachino and K. J. Waldron, “Copper tolerance in bacteria requires the activation of multiple
1067 accessory pathways,” *Molecular Microbiology*, vol. 114, no. 3, pp. 377–390, Sep. 2020, doi:
1068 10.1111/mmi.14522.
1069 [92] A. Janssens *et al.*, “SlyB encapsulates outer membrane proteins in stress-induced lipid
1070 nanodomains,” *Nature*, vol. 626, no. 7999, pp. 617–625, Feb. 2024, doi: 10.1038/s41586-023-
1071 06925-5.
1072 [93] Z. Lin *et al.*, “Virulence and Stress Responses of Shigella flexneri Regulated by PhoP/PhoQ,” *Front.*
1073 *Microbiol.*, vol. 8, p. 2689, Jan. 2018, doi: 10.3389/fmicb.2017.02689.
1074 [94] B. Yang *et al.*, “Identification of Novel phoP-phoQ Regulated Genes that Contribute to Polymyxin B
1075 Tolerance in Pseudomonas aeruginosa,” *Microorganisms*, vol. 9, no. 2, p. 344, Feb. 2021, doi:
1076 10.3390/microorganisms9020344.
1077 [95] P. A. Queiroz *et al.*, “Proteomic profiling of *Klebsiella pneumoniae* carbapenemase (KPC)-producer
1078 *Klebsiella pneumoniae* after induced polymyxin resistance,” *Future Microbiology*, vol. 16, no. 15,
1079 pp. 1195–1207, Oct. 2021, doi: 10.2217/fmb-2021-0005.
1080 [96] T. Uehara, K. Suefuji, N. Valbuena, B. Meehan, M. Donegan, and J. T. Park, “Recycling of the
1081 Anhydro- *N* -Acetylmuramic Acid Derived from Cell Wall Murein Involves a Two-Step Conversion to
1082 *N* -Acetylglucosamine-Phosphate,” *J Bacteriol*, vol. 187, no. 11, pp. 3643–3649, Jun. 2005, doi:
1083 10.1128/JB.187.11.3643-3649.2005.
1084 [97] S. Harris *et al.*, “Variant O89 O-Antigen of E. coli Is Associated With Group 1 Capsule Loci and
1085 Multidrug Resistance,” *Front. Microbiol.*, vol. 9, p. 2026, Aug. 2018, doi:
1086 10.3389/fmicb.2018.02026.

1087 [98] E. Pradel, C. T. Parker, and C. A. Schnaitman, “Structures of the rfaB, rfaI, rfaJ, and rfaS genes of
1088 Escherichia coli K-12 and their roles in assembly of the lipopolysaccharide core,” *J Bacteriol*, vol.
1089 174, no. 14, pp. 4736–4745, Jul. 1992, doi: 10.1128/jb.174.14.4736-4745.1992.
1090 [99] J. Mortier *et al.*, “Gene Erosion Can Lead to Gain-of-Function Alleles That Contribute to Bacterial
1091 Fitness,” *mBio*, vol. 12, no. 4, pp. e01129-21, Aug. 2021, doi: 10.1128/mBio.01129-21.
1092 [100] N. S. Wigginton *et al.*, “Binding of Silver Nanoparticles to Bacterial Proteins Depends on Surface
1093 Modifications and Inhibits Enzymatic Activity,” *Environ. Sci. Technol.*, vol. 44, no. 6, pp. 2163–2168,
1094 Mar. 2010, doi: 10.1021/es903187s.
1095 [101] G. Li and K. D. Young, “A new suite of tnaA mutants suggests that Escherichia coli tryptophanase is
1096 regulated by intracellular sequestration and by occlusion of its active site,” *BMC Microbiol*, vol. 15,
1097 no. 1, p. 14, Dec. 2015, doi: 10.1186/s12866-015-0346-3.
1098 [102] Wm. A. Newton and E. E. Snell, “CATALYTIC PROPERTIES OF TRYPTOPHANASE, A
1099 MULTIFUNCTIONAL PYRIDOXAL PHOSPHATE ENZYME,” *Proc. Natl. Acad. Sci. U.S.A.*, vol. 51, no. 3,
1100 pp. 382–389, Mar. 1964, doi: 10.1073/pnas.51.3.382.
1101 [103] N. Awano, M. Wada, H. Mori, S. Nakamori, and H. Takagi, “Identification and Functional Analysis of
1102 *Escherichia coli* Cysteine Desulfhydrases,” *Appl Environ Microbiol*, vol. 71, no. 7, pp. 4149–4152,
1103 Jul. 2005, doi: 10.1128/AEM.71.7.4149-4152.2005.
1104 [104] N. Awano, M. Wada, A. Kohdoh, T. Oikawa, H. Takagi, and S. Nakamori, “Effect of cysteine
1105 desulfhydrase gene disruption on l-cysteine overproduction in Escherichia coli,” *Applied*
1106 *Microbiology and Biotechnology*, vol. 62, no. 2–3, pp. 239–243, Aug. 2003, doi: 10.1007/s00253-
1107 003-1262-2.
1108 [105] K. Li *et al.*, “Escherichia coli Uses Separate Enzymes to Produce H2S and Reactive Sulfane Sulfur
1109 From L-cysteine,” *Front. Microbiol.*, vol. 10, p. 298, Feb. 2019, doi: 10.3389/fmicb.2019.00298.
1110 [106] Y. Zhou and J. A. Imlay, “Escherichia coli Uses a Dedicated Importer and Desulfidase To Ferment
1111 Cysteine,” *mBio*, vol. 13, no. 2, pp. e02965-21, Apr. 2022, doi: 10.1128/mbio.02965-21.
1112 [107] D. R. X. Lim, Y. Chen, L. F. Ng, J. Gruber, and Y. Gan, “Glutathione catabolism by *Enterobacteriaceae*
1113 species to hydrogen sulphide adversely affects the viability of host systems in the presence of
1114 5’fluorodeoxyuridine,” *Molecular Microbiology*, vol. 117, no. 5, pp. 1089–1103, May 2022, doi:
1115 10.1111/mmi.14893.
1116 [108] M. E. Starodub and J. T. Trevors, “Silver accumulation and resistance in Escherichia coli R1,”
1117 *Journal of Inorganic Biochemistry*, vol. 39, no. 4, pp. 317–325, Aug. 1990, doi: 10.1016/0162-
1118 0134(90)80030-2.
1119 [109] Z. Huang *et al.*, “Alleviation of heavy metal and silver nanoparticle toxicity and enhancement of
1120 their removal by hydrogen sulfide in Phanerochaete chrysosporium,” *Chemosphere*, vol. 224, pp.
1121 554–561, Jun. 2019, doi: 10.1016/j.chemosphere.2019.02.190.
1122 [110] K. Shatalin, E. Shatalina, A. Mironov, and E. Nudler, “H_2S: A Universal Defense Against Antibiotics
1123 in Bacteria,” *Science*, vol. 334, no. 6058, pp. 986–990, Nov. 2011, doi: 10.1126/science.1209855.
1124 [111] Y. Lin *et al.*, “Periplasmic biomineralization for semi-artificial photosynthesis,” *Sci. Adv.*, vol. 9, no.
1125 29, p. eadg5858, Jul. 2023, doi: 10.1126/sciadv.adg5858.
1126 [112] L. C. Reimer, J. Sarda Carbasse, J. Koblitz, A. Podstawka, and J. Overmann, “Escherichia coli (Migula
1127 1895) Castellani and Chalmers 1919.” DSMZ, May 10, 2023. doi:
1128 10.13145/BACDIVE4433.20230509.8.
1129 [113] T. H. Han, J.-H. Lee, M. H. Cho, T. K. Wood, and J. Lee, “Environmental factors affecting indole
1130 production in Escherichia coli,” *Research in Microbiology*, vol. 162, no. 2, pp. 108–116, Feb. 2011,
1131 doi: 10.1016/j.resmic.2010.11.005.
1132 [114] J. Zhang, C. Quan, C. Wang, H. Wu, Z. Li, and Q. Ye, “Systematic manipulation of glutathione
1133 metabolism in Escherichia coli for improved glutathione production,” *Microb Cell Fact*, vol. 15, no.
1134 1, p. 38, Dec. 2016, doi: 10.1186/s12934-016-0439-1.

[115] C. E. Deutch, I. Spahija, and C. E. Wagner, “Susceptibility of *Escherichia coli* to the toxic L-proline analogue L-selenaproline is dependent on two L-cystine transport systems,” *J Appl Microbiol*, vol. 117, no. 5, pp. 1487–1499, Nov. 2014, doi: 10.1111/jam.12623.
[116] S. Korshunov, K. R. C. Imlay, and J. A. Imlay, “Cystine import is a valuable but risky process whose hazards *Escherichia coli* minimizes by inducing a cysteine exporter,” *Molecular Microbiology*, vol. 113, no. 1, pp. 22–39, Jan. 2020, doi: 10.1111/mmi.14403.
[117] S. Yamazaki, K. Takei, and G. Nonaka, “*ydjN* encodes an *S* -sulfocysteine transporter required by *Escherichia coli* for growth on *S* -sulfocysteine as a sulfur source,” *FEMS Microbiology Letters*, vol. 363, no. 17, p. fnw185, Sep. 2016, doi: 10.1093/femsle/fnw185.
[118] I. Ohtsu *et al.*, “Uptake of L-cystine via an ABC transporter contributes defense of oxidative stress in the L-cystine export-dependent manner in Escherichia coli,” *PLoS ONE*, vol. 10, no. 4, p. e0120619, Apr. 2015, doi: 10.1371/journal.pone.0120619.
[119] V. Pareek *et al.*, “Does Silver in Different Forms Affect Bacterial Susceptibility and Resistance? A Mechanistic Perspective,” *ACS Appl. Bio Mater.*, vol. 5, no. 2, pp. 801–817, Feb. 2022, doi: 10.1021/acsabm.1c01179.
[120] K. Yamamoto and A. Ishihama, “Transcriptional Response of *Escherichia coli* to External Zinc,” *J Bacteriol*, vol. 187, no. 18, pp. 6333–6340, Sep. 2005, doi: 10.1128/JB.187.18.6333-6340.2005.
[121] K. Yamamoto and A. Ishihama, “Transcriptional response of *Escherichia coli* to external copper,” *Molecular Microbiology*, vol. 56, no. 1, pp. 215–227, Apr. 2005, doi: 10.1111/j.1365-2958.2005.04532.x.
[122] Y. Xia *et al.*, “Sulfide production and oxidation by heterotrophic bacteria under aerobic conditions,” *The ISME Journal*, vol. 11, no. 12, pp. 2754–2766, Dec. 2017, doi: 10.1038/ismej.2017.125.
[123] S. Han, Y. Li, and H. Gao, “Generation and Physiology of Hydrogen Sulfide and Reactive Sulfur Species in Bacteria,” *Antioxidants*, vol. 11, no. 12, p. 2487, Dec. 2022, doi: 10.3390/antiox11122487.
[124] H.-M. Park *et al.*, “Structures of the γ-class carbonic anhydrase homologue YrdA suggest a possible allosteric switch,” *Acta Crystallogr D Biol Crystallogr*, vol. 68, no. 8, pp. 920–926, Aug. 2012, doi: 10.1107/S0907444912017210.
[125] E. Yohannes, A. E. Thurber, J. C. Wilks, D. P. Tate, and J. L. Slonczewski, “Polyamine stress at high pH in Escherichia coli K-12,” *BMC Microbiol*, vol. 5, no. 1, p. 59, Dec. 2005, doi: 10.1186/1471-2180-5-59.
[126] C. Merlin, M. Masters, S. McAteer, and A. Coulson, “Why Is Carbonic Anhydrase Essential to *Escherichia coli* ?,” *J Bacteriol*, vol. 185, no. 21, pp. 6415–6424, Nov. 2003, doi: 10.1128/JB.185.21.6415-6424.2003.
[127] S. Hayashi, K. Itoh, and K. Suyama, “Genes of Bacillus subtilis 168 that Support Growth of the Cyanobacterium, Synechococcus leopoliensis CCAP1405/1 on Agar Media,” *Microb Ecol*, vol. 70, no. 3, pp. 849–852, Oct. 2015, doi: 10.1007/s00248-015-0610-y.
[128] A. Miseta and P. Csutora, “Relationship Between the Occurrence of Cysteine in Proteins and the Complexity of Organisms,” *Molecular Biology and Evolution*, vol. 17, no. 8, pp. 1232–1239, Aug. 2000, doi: 10.1093/oxfordjournals.molbev.a026406.
[129] H. Alhazmi, “FT-IR Spectroscopy for the Identification of Binding Sites and Measurements of the Binding Interactions of Important Metal Ions with Bovine Serum Albumin,” *Sci. Pharm.*, vol. 87, no. 1, p. 5, Feb. 2019, doi: 10.3390/scipharm87010005.
[130] P. P. Jing, Y. X. Li, Y. H. Su, W. L. Liang, and Y. X. Leng, “The role of metal ions in the behavior of bovine serum albumin molecules under physiological environment,” *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 267, p. 120604, Feb. 2022, doi: 10.1016/j.saa.2021.120604.

1183 [131] V. Mishra and R. J. Heath, “Structural and Biochemical Features of Human Serum Albumin Essential
1184 for Eukaryotic Cell Culture,” *IJMS*, vol. 22, no. 16, p. 8411, Aug. 2021, doi: 10.3390/ijms22168411.
1185 [132] N. Reznik *et al.*, “Intestinal mucin is a chaperone of multivalent copper,” *Cell*, vol. 185, no. 22, pp.
1186 4206-4215.e11, Oct. 2022, doi: 10.1016/j.cell.2022.09.021.
1187 [133] Q. Duan, M. Zhou, L. Zhu, and G. Zhu, “Flagella and bacterial pathogenicity,” *J. Basic Microbiol.*, vol.
1188 53, no. 1, pp. 1–8, Jan. 2013, doi: 10.1002/jobm.201100335.
1189 [134] A. Bougdour, S. Wickner, and S. Gottesman, “Modulating RssB activity: IraP, a novel regulator of σ^S
1190 stability in *Escherichia coli*,” *Genes Dev.*, vol. 20, no. 7, pp. 884–897, Apr. 2006, doi:
1191 10.1101/gad.1400306.
1192 [135] F. Fontaine, E. J. Stewart, A. B. Lindner, and F. Taddei, “Mutations in two global regulators lower
1193 individual mortality in *Escherichia coli*,” *Molecular Microbiology*, vol. 67, no. 1, pp. 2–14, Jan. 2008,
1194 doi: 10.1111/j.1365-2958.2007.05988.x.
1195 [136] D. Schneider, W. Kaiser, C. Stutz, A. Holinski, O. Mayans, and P. Babinger, “YbiB from Escherichia
1196 coli, the Defining Member of the Novel TrpD2 Family of Prokaryotic DNA-binding Proteins,” *Journal*
1197 *of Biological Chemistry*, vol. 290, no. 32, pp. 19527–19539, Aug. 2015, doi:
1198 10.1074/jbc.M114.620575.
1199 [137] M. P. Thorgersen *et al.*, “Deciphering Microbial Metal Toxicity Responses via Random Bar Code
1200 Transposon Site Sequencing and Activity-Based Metabolomics,” *Appl Environ Microbiol*, vol. 87, no.
1201 21, pp. e01037-21, Oct. 2021, doi: 10.1128/AEM.01037-21.
1202 [138] K. F. S. Luk, A. H. Maki, and R. J. Hoover, “Studies of heavy metal binding with polynucleotides
1203 using optical detection of magnetic resonance. Silver(I) binding,” *J. Am. Chem. Soc.*, vol. 97, no. 5,
1204 pp. 1241–1242, Mar. 1975, doi: 10.1021/ja00838a047.
1205 [139] B. Giese and D. McNaughton, “Surface-Enhanced Raman Spectroscopic and Density Functional
1206 Theory Study of Adenine Adsorption to Silver Surfaces,” *J. Phys. Chem. B*, vol. 106, no. 1, pp. 101–
1207 112, Jan. 2002, doi: 10.1021/jp010789f.
1208 [140] J. Norambuena *et al.*, “Copper ions inhibit pentose phosphate pathway function in Staphylococcus
1209 aureus,” *PLoS Pathog*, vol. 19, no. 5, p. e1011393, May 2023, doi: 10.1371/journal.ppat.1011393.
1210 [141] C. Molteni, H. K. Abicht, and M. Solioz, “Killing of Bacteria by Copper Surfaces Involves Dissolved
1211 Copper,” *AEM*, vol. 76, no. 12, pp. 4099–4101, Jun. 2010, doi: 10.1128/AEM.00424-10.
1212 [142] S. L. Warnes, S. M. Green, H. T. Michels, and C. W. Keevil, “Biocidal Efficacy of Copper Alloys
1213 against Pathogenic Enterococci Involves Degradation of Genomic and Plasmid DNAs,” *AEM*, vol. 76,
1214 no. 16, pp. 5390–5401, Aug. 2010, doi: 10.1128/AEM.03050-09.
1215 [143] N. Nordholt, O. Kanaris, S. B. I. Schmidt, and F. Schreiber, “Persistence against benzalkonium
1216 chloride promotes rapid evolution of tolerance during periodic disinfection,” *Nat Commun*, vol. 12,
1217 no. 1, p. 6792, Dec. 2021, doi: 10.1038/s41467-021-27019-8.
1218 [144] H. Kaur, M. Rosenberg, M. Kook, D. Danilian, V. Kisand, and A. Ivask, “Antibacterial activity of solid
1219 surfaces is critically dependent on relative humidity, inoculum volume and organic soiling,”
1220 Microbiology, preprint, Mar. 2023. doi: 10.1101/2023.03.28.534510.
1221 [145] G. Grézal *et al.*, “Plasticity and Stereotypic Rewiring of the Transcriptome Upon Bacterial Evolution
1222 of Antibiotic Resistance,” *Molecular Biology and Evolution*, vol. 40, no. 2, p. msad020, Feb. 2023,
1223 doi: 10.1093/molbev/msad020.
1224 [146] C. Espírito Santo, P. V. Morais, and G. Grass, “Isolation and Characterization of Bacteria Resistant
1225 to Metallic Copper Surfaces,” *Appl Environ Microbiol*, vol. 76, no. 5, pp. 1341–1348, Mar. 2010, doi:
1226 10.1128/AEM.01952-09.
1227 [147] E. F. S. Vieira, J. de A. Simoni, and C. Airoldi, “Interaction of cations with SH-modified silica gel:
1228 thermochemical study through calorimetric titration and direct extent of reaction determination,”
1229 *J. Mater. Chem.*, vol. 7, no. 11, pp. 2249–2252, 1997, doi: 10.1039/a704286h.

1230 [148] H. D. Betts, C. Whitehead, and H. H. Harris, “Silver in biology and medicine: opportunities for
1231 metallomics researchers,” *Metallomics*, vol. 13, no. 1, p. mfaa001, Jan. 2021, doi:
1232 10.1093/mtomcs/mfaa001.
1233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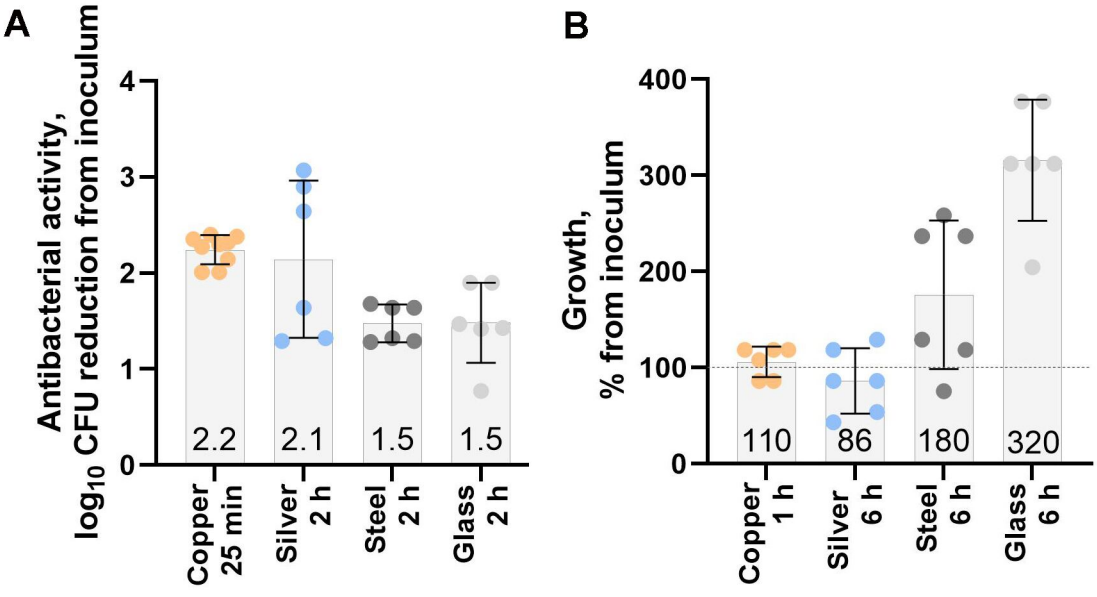
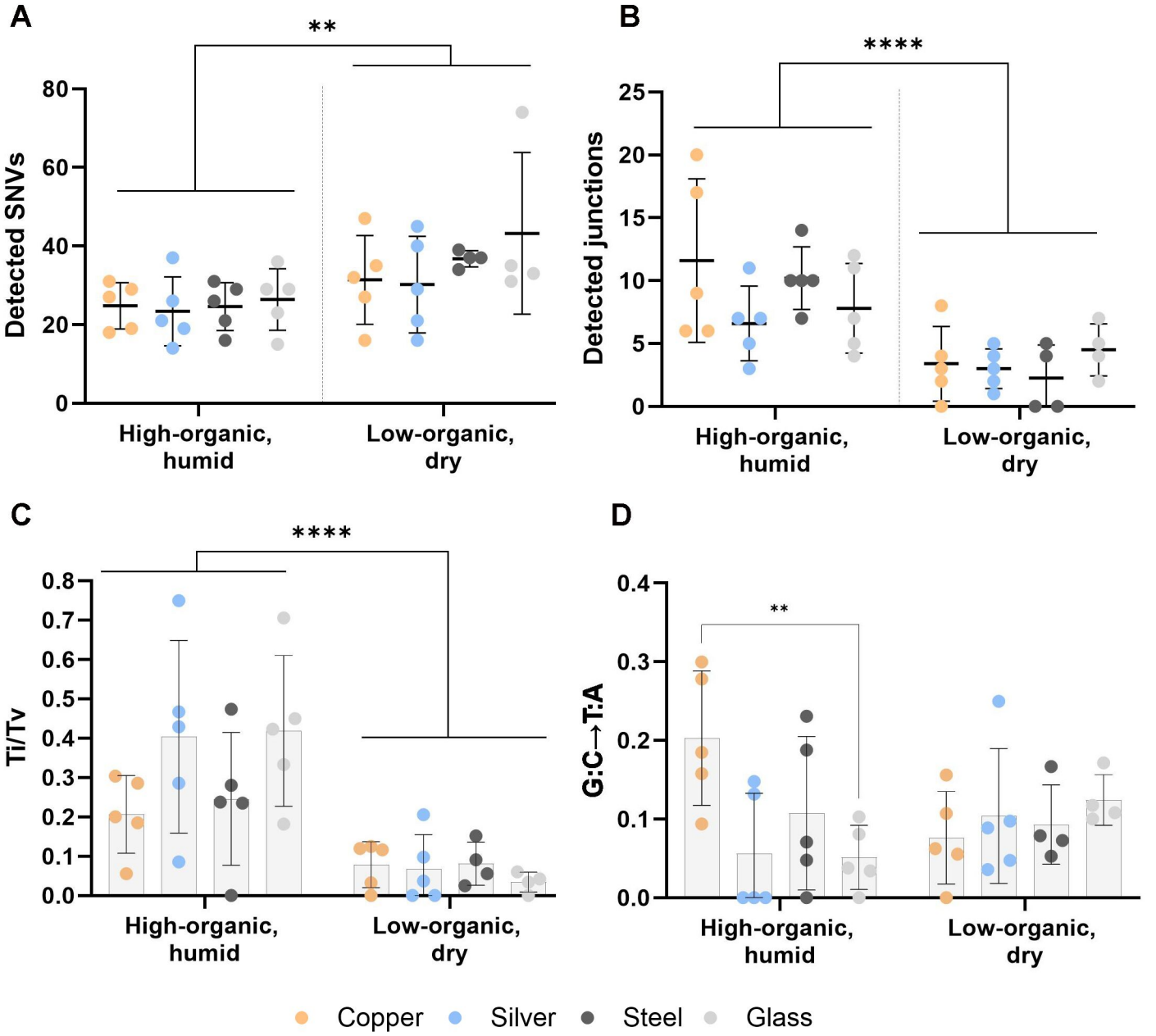
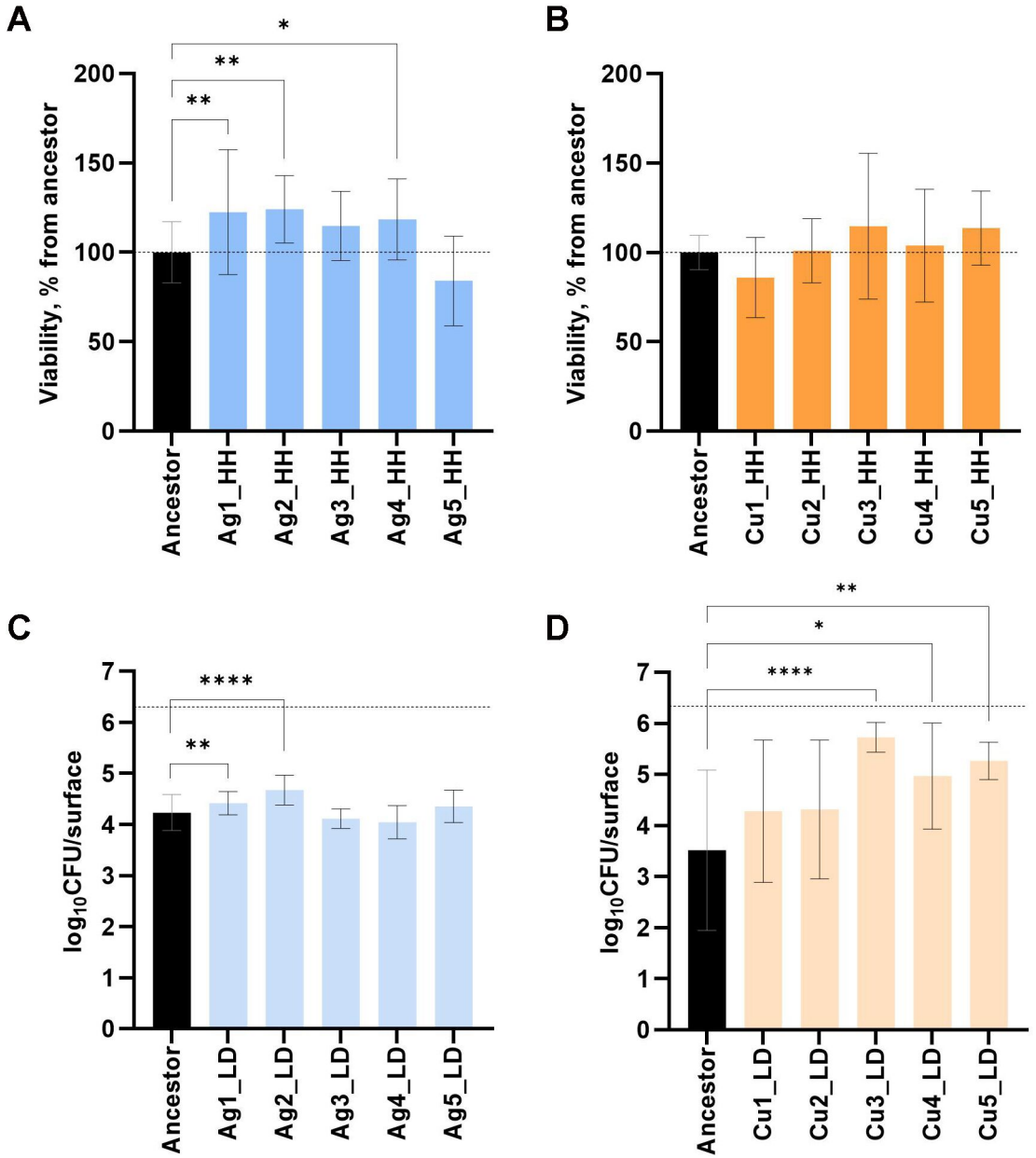
mntP
borD
basS
kdpD
kup
basR
sodA
gshA
mgtA
mntS
arnE
mgrB
ibpB
ftnA
arnF
arnT
mgtL
arnD

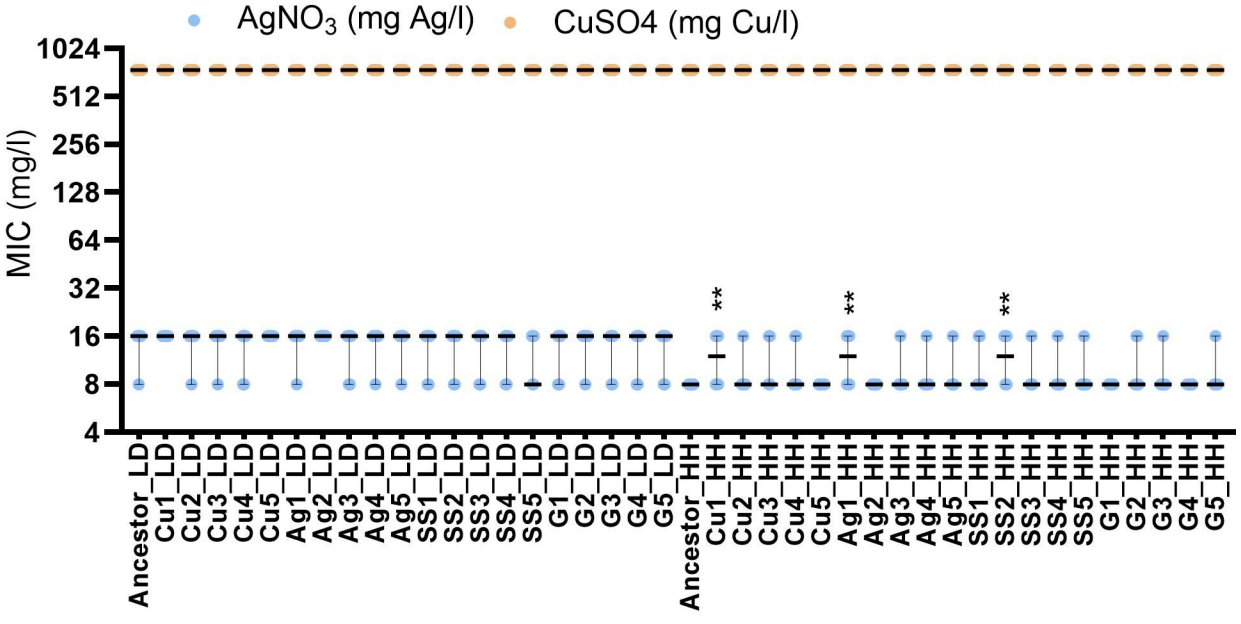

A
Antibacterial activity,
log10 CFU reduction from inoculum
4
3
2
1
0
2.2
2.1
1.5
1.5
Copper 25 min
Silver 2 h
Steel 2 h
Glass 2 h
B
Growth,
% from inoculum
400
300
200
100
0
110
86
180
320
Copper 1 h
Silver 6 h
Steel 6 h
Glass 6 h

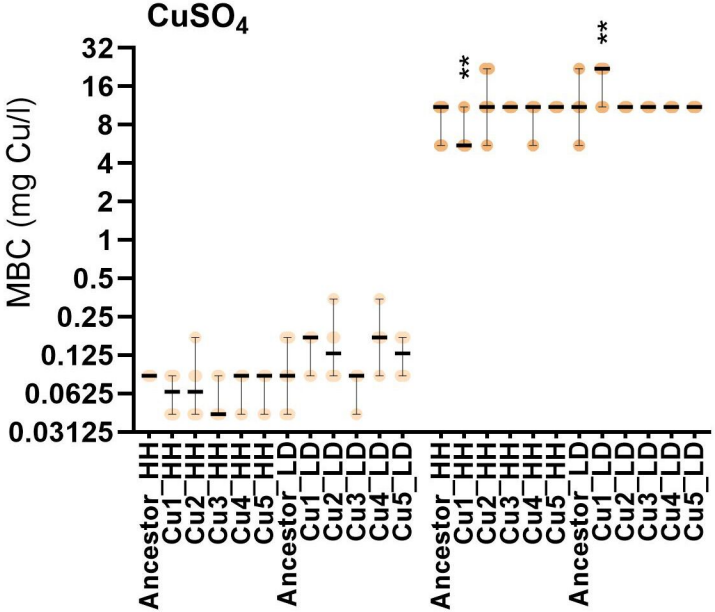

A
**
Detected SNVs
80
60
40
20
0
High-organic, humid
Low-organic, dry
B
****
Detected junctions
25
20
15
10
5
0
High-organic, humid
Low-organic, dry
C
****
Ti/Tv
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
High-organic, humid
Low-organic, dry
D
**
G:C→T:A
0.4
0.3
0.2
0.1
0.0
High-organic, humid
Low-organic, dry
Copper
Silver
Steel
Glass

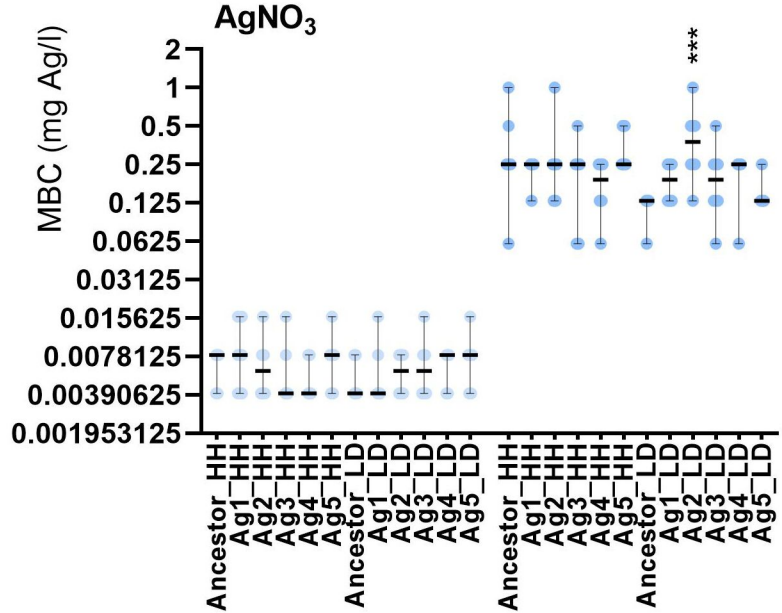
	Ag LD	Cu LD	Ag HH	Cu HH	SS LD	G LD	SS HH	G HH
Amino acid metabolic process		1	5				2	1
Carbohydrate derivative metabolic process	3			1	1			
Carbohydrate metabolic process	2			1	1		2	5
Cell adhesion							2	5
Cell motility	1					2		
Cell projection organization						1		
Chromosome segregation					1			
Cytokinesis					1			
DNA recombination							2	
DNA repair							1	1
DNA replication							2	5
Generation of precursor metabolites and energy	1				1		1	
Lipid metabolic process				1	1			
Nucleobase-containing small molecule metabolic process	1				1			
Protein catabolic process							5	5
Protein folding	1				1			
Protein-containing complex assembly	1				1		1	1
Regulation of DNA-templated transcription		2			1		5	5
Response to stimulus	4	1	2		2	1	5	5
RNA metabolic process							2	5
Signaling	1	1						
Small molecule biosynthetic process	2	1			1		1	1
Sulfur compound metabolic process		1			1		2	3
Translation		1	1					
Transport	2		1		2	1	3	5

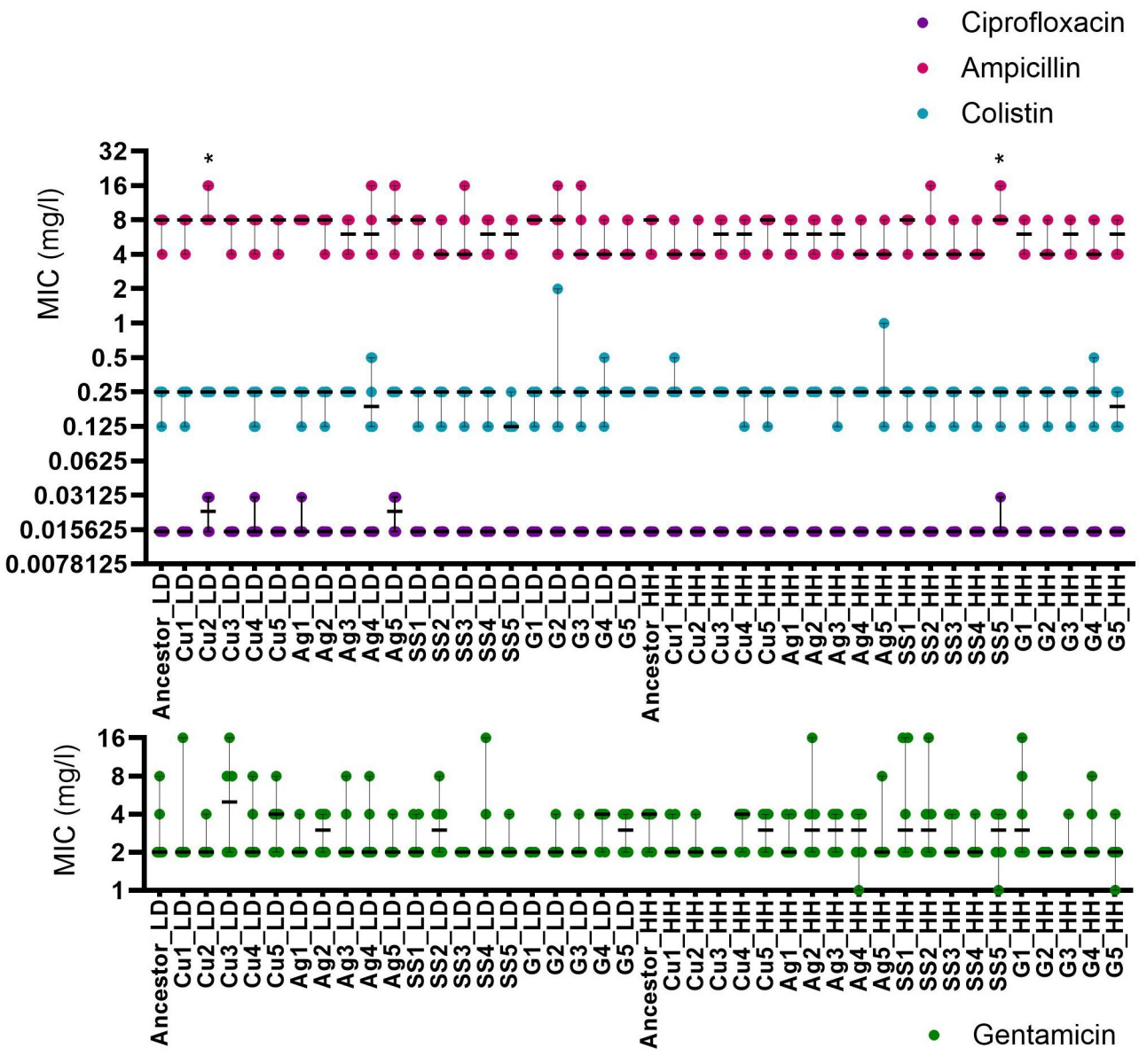
	Ag LD	Cu LD	Ag HH	Cu HH	SS LD	G LD	SS HH	G HH
Response to stress								
Cellular response to cell envelope stress	■							
Response to osmotic stress	■					■		
Cellular response to starvation	■						■	■
Response to starvation							■	■
DNA damage response	■						■	■
Response to oxidative stress			■		■			
Cellular response to chemical stress			■				■	
Response to heat					■		■	■
Response to inorganic substance and metal ion								
Cellular response to metal ion	■		■					
Response to zinc ion	■							
Response to lead ion	■							
Response to cadmium ion			■					
Response to hydrogen peroxide			■					
Sulfur metabolism								
Hydrogen sulfide biosynthetic process		■						
Sulfur compound metabolic process		■			■			
Sulfate assimilation		■						
Sulfur utilization			■				■	■
Sulfur amino acid transport			■					
Sulfur compound biosynthetic process					■		■	■
Sulfur amino acid catabolic process							■	■
Sulfur compound metabolic process							■	■
Cellular response to sulfur starvation							■	■
Barriers (cell wall, LPS, slime capsule, biofilm)								
Colanic acid metabolic process	■							
Peptidoglycan metabolic process	■							
Biofilm formation			■				■	■
Lipopolysaccharide biosynthetic process				■				

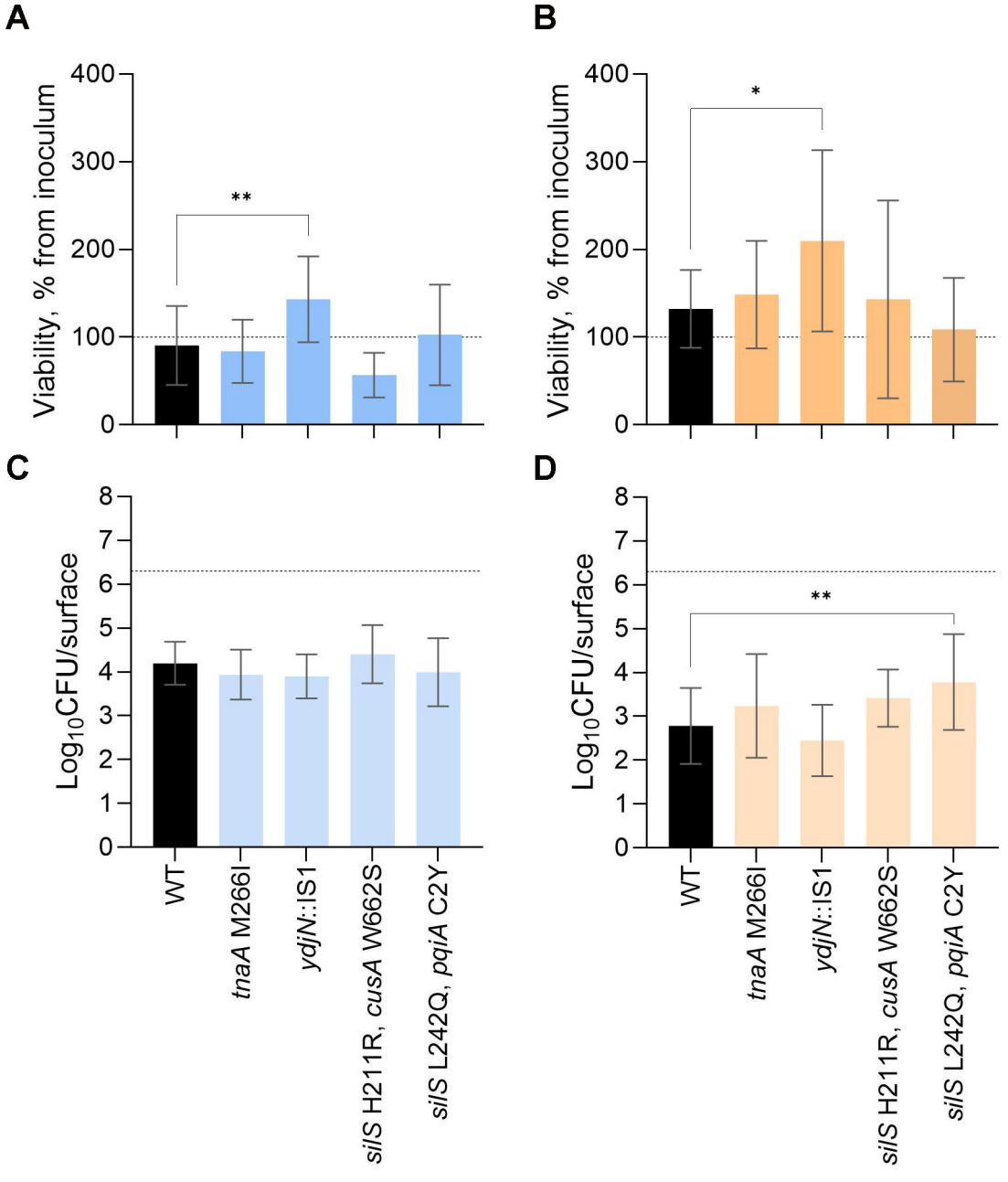

A
B
C
D
Viability, % from ancestor
log10CFU/surface
Ancestor
Ag1_HH
Ag2_HH
Ag3_HH
Ag4_HH
Ag5_HH
Cu1_HH
Cu2_HH
Cu3_HH
Cu4_HH
Cu5_HH
Ag1_LD
Ag2_LD
Ag3_LD
Ag4_LD
Ag5_LD
Cu1_LD
Cu2_LD
Cu3_LD
Cu4_LD
Cu5_LD
*
**
****

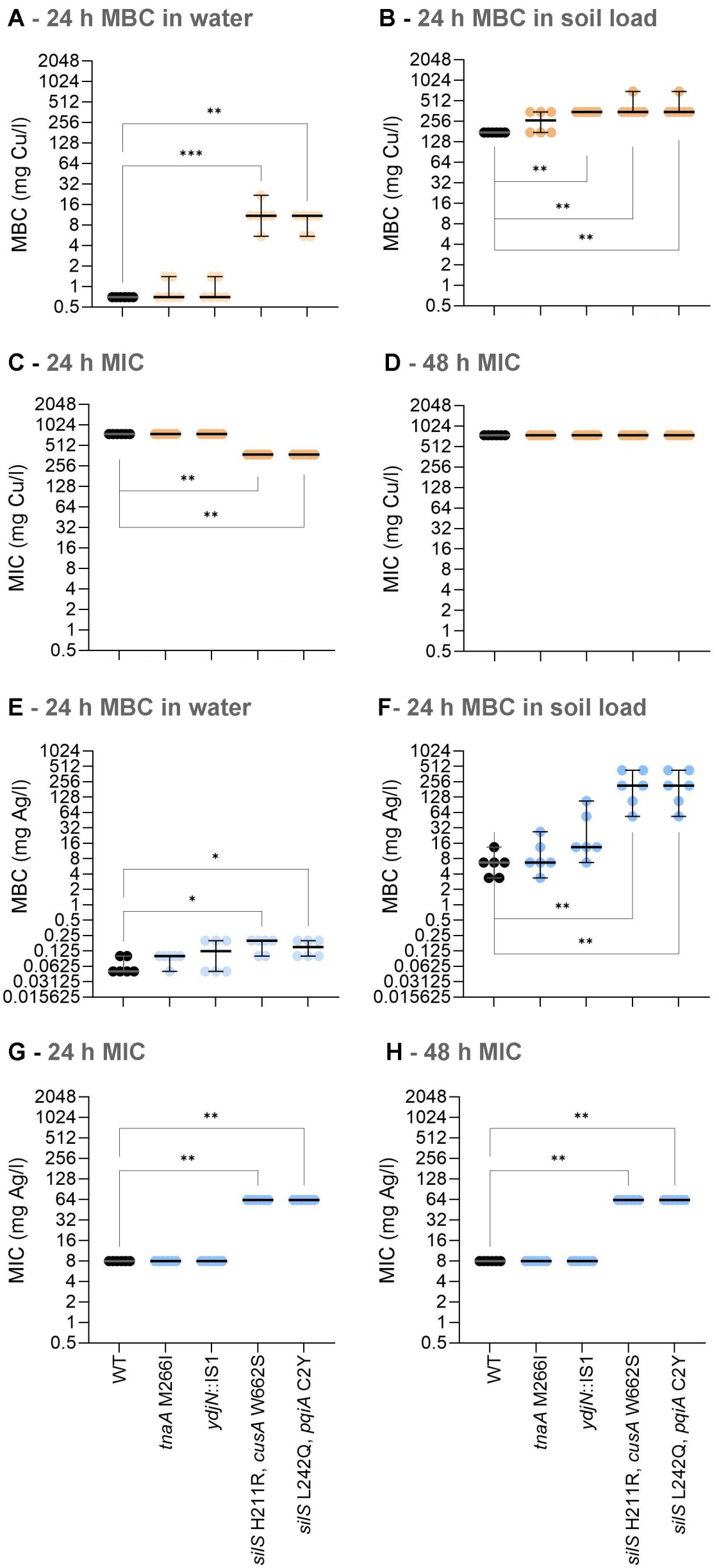

AgNO3 (mg Ag/l)
CuSO4 (mg Cu/l)
MIC (mg/l)
1024
512
256
128
64
32
16
8
4
**
**
**
Ancestor_LD
Cu1_LD
Cu2_LD
Cu3_LD
Cu4_LD
Cu5_LD
Ag1_LD
Ag2_LD
Ag3_LD
Ag4_LD
Ag5_LD
SS1_LD
SS2_LD
SS3_LD
SS4_LD
SS5_LD
G1_LD
G2_LD
G3_LD
G4_LD
G5_LD
Ancestor_HH
Cu1_HH
Cu2_HH
Cu3_HH
Cu4_HH
Cu5_HH
Ag1_HH
Ag2_HH
Ag3_HH
Ag4_HH
Ag5_HH
SS1_HH
SS2_HH
SS3_HH
SS4_HH
SS5_HH
G1_HH
G2_HH
G3_HH
G4_HH
G5_HH

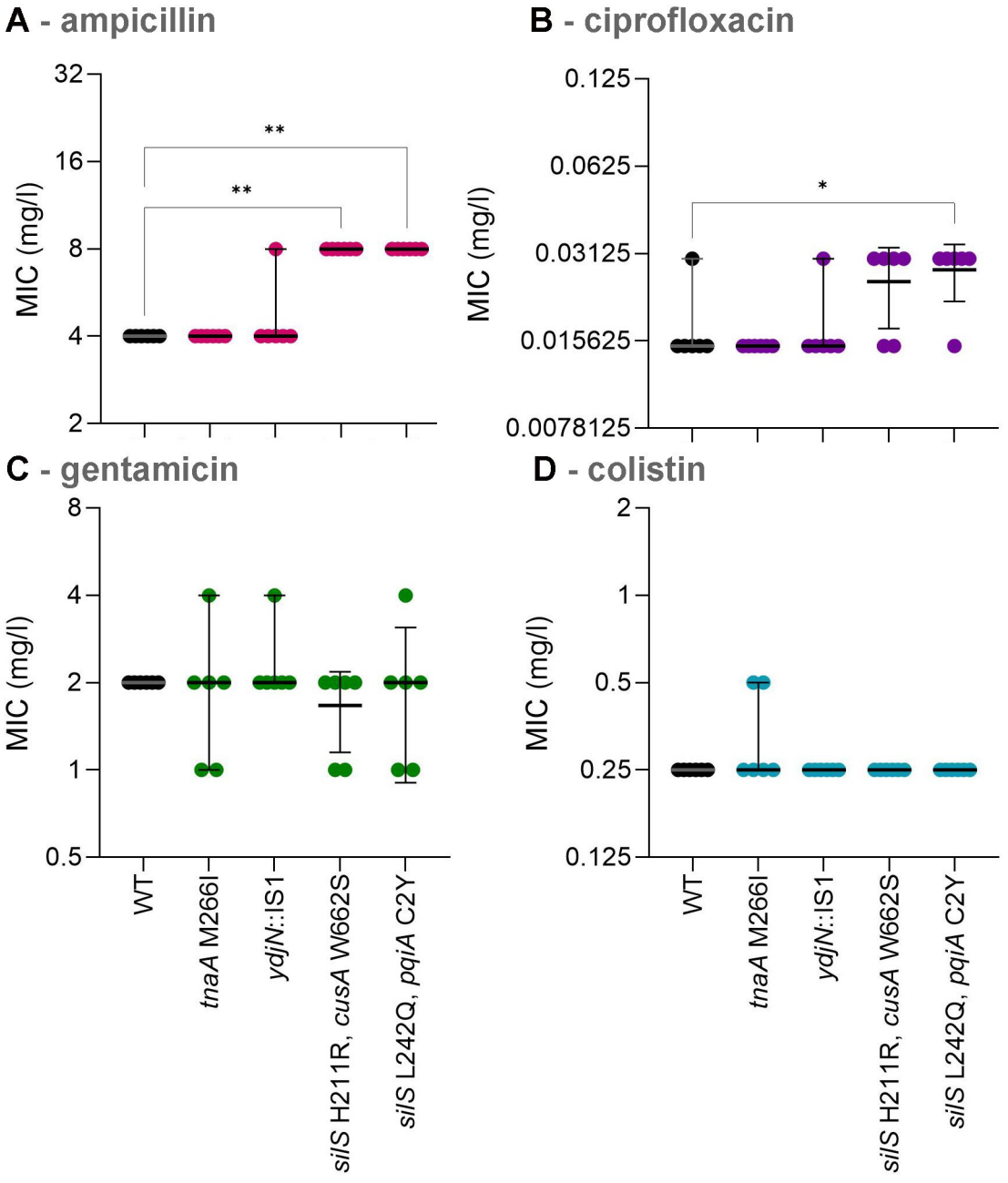

CuSO4
MBC (mg Cu/l)
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
**
**
Ancestor_HH
Cu1_HH
Cu2_HH
Cu3_HH
Cu4_HH
Cu5_HH
Ancestor_LD
Cu1_LD
Cu2_LD
Cu3_LD
Cu4_LD
Cu5_LD
Ancestor_HH
Cu1_HH
Cu2_HH
Cu3_HH
Cu4_HH
Cu5_HH
Ancestor_LD
Cu1_LD
Cu2_LD
Cu3_LD
Cu4_LD
Cu5_LD
Low-organic exposure
High-organic exposure


AgNO3
MBC (mg Ag/l)
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
0.0078125
0.00390625
0.001953125
***
Ancestor_HH
Ag1_HH
Ag2_HH
Ag3_HH
Ag4_HH
Ag5_HH
Ancestor_LD
Ag1_LD
Ag2_LD
Ag3_LD
Ag4_LD
Ag5_LD
Ancestor_HH
Ag1_HH
Ag2_HH
Ag3_HH
Ag4_HH
Ag5_HH
Ancestor_LD
Ag1_LD
Ag2_LD
Ag3_LD
Ag4_LD
Ag5_LD
Low-organic exposure
High-organic exposure


Ciprofloxacin
Ampicillin
Colistin
MIC (mg/l)
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
0.0078125
*
*
Ancestor_LD
Cu1_LD
Cu2_LD
Cu3_LD
Cu4_LD
Cu5_LD
Ag1_LD
Ag2_LD
Ag3_LD
Ag4_LD
Ag5_LD
SS1_LD
SS2_LD
SS3_LD
SS4_LD
SS5_LD
G1_LD
G2_LD
G3_LD
G4_LD
G5_LD
Ancestor_HH
Cu1_HH
Cu2_HH
Cu3_HH
Cu4_HH
Cu5_HH
Ag1_HH
Ag2_HH
Ag3_HH
Ag4_HH
Ag5_HH
SS1_HH
SS2_HH
SS3_HH
SS4_HH
SS5_HH
G1_HH
G2_HH
G3_HH
G4_HH
G5_HH
MIC (mg/l)
16
8
4
2
1
Gentamicin


A
B
C
D
Viability, % from inoculum
Log10CFU/surface
400
300
200
100
0
8
7
6
5
4
3
2
1
0
**
*
**
WT
tnaA M266I
ydjN::IS1
silS H211R, cusA W662S
silS L242Q, pqiA C2Y


A - 24 h MBC in water
B - 24 h MBC in soil load
C - 24 h MIC
D - 48 h MIC
E - 24 h MBC in water
F- 24 h MBC in soil load
G - 24 h MIC
H - 48 h MIC
MBC (mg Cu/l)
MIC (mg Cu/l)
MBC (mg Ag/l)
MIC (mg Ag/l)
2048
1024
512
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
*
**
***
WT
tnaA M266I
ydjN::IS1
silS H211R, cusA W662S
silS L242Q, pqiA C2Y


A - ampicillin
B - ciprofloxacin
C - gentamicin
D - colistin
MIC (mg/l)
32
16
8
4
2
**
**
0.125
0.0625
0.03125
0.015625
0.0078125
*
8
4
2
1
0.5
2
1
0.5
0.25
0.125
WT
tnaA M266I
ydjN::IS1
silS H211R, cusA W662S
silS L242Q, pqiA C2Y