Sub-MIC kill kinetics of copper and silver in *Escherichia coli*

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Abstract

Copper and silver are often considered to share antibacterial mechanisms and defense responses. Here, we show that at sub-MIC concentrations, copper gradually inhibits the growth of *Escherichia coli*, while silver appears to extend the lag phase through initial killing followed by normal regrowth. The distinct kinetics indicate that copper and silver may differentially influence tolerance and resistance development, with implications for risk assessment and the design of metal-based antimicrobial applications.

Main text

Silver and copper are widely used in various antimicrobial applications with the intent to kill or limit the spread of potentially pathogenic microbes. Unlike toxic silver, copper is an essential micronutrient tightly regulated by homeostasis but becomes toxic in excess (*1*). Their mechanism of action and corresponding bacterial defenses are often considered overlapping due to similar coordination chemistry and sulfhydryl reactivity, leading to shared efflux and sequestration mechanisms (*1–4*). A key difference is Cu(I)/Cu(II) redox activity, which generates reactive oxygen species and causes direct damage such as lipid peroxidation (*5*). We have previously found that silver and copper also differ in their relative toxicity to bacteria depending on exposure conditions. *E. coli* tolerates higher copper ion concentrations (millimolar range) than silver ion concentrations (micromolar range) in liquid exposure media but survives longer on silver surfaces than on copper surfaces in semi-dry exposure conditions (*6*, *7*). These contrasts suggest distinct mechanisms or condition-dependent defense responses.

Antibacterial activity is typically measured as minimal inhibitory (MIC) or bactericidal (MBC) concentrations (*8*). MIC indicates presence or absence of growth after standardized exposure while MBC only reflects endpoint mortality (>99.9%). Neither captures dynamics, where an antimicrobial agent is detoxified or kills part of the population allowing survivors to regrow before the assay endpoint. For that level of comparison, growth and kill kinetics assays in well-defined experimental conditions should be used. Implications of the strain, media and exposure conditions selection on the results of the current study are discussed in Supplementary Note 1.

To study the effects of copper and silver on the growth and viability of *E. coli*, bacteria were exposed to concentration increments of respective salts in buffered minimal medium. At comparable sub-MIC concentration ranges, $CuSO_4$ increased doubling time and reduced biomass yield of *E. coli* in a dose-dependent manner (Fig. 1A,

Supplementary Fig. S1). In contrast, $AgNO_3$ seemingly prolonged lag time at population level with little effect on doubling time or yield (Fig. 1B, Supplementary Fig. S1). These patterns indicated distinct bacterial responses to inhibitory sub-MIC concentrations of copper and silver.

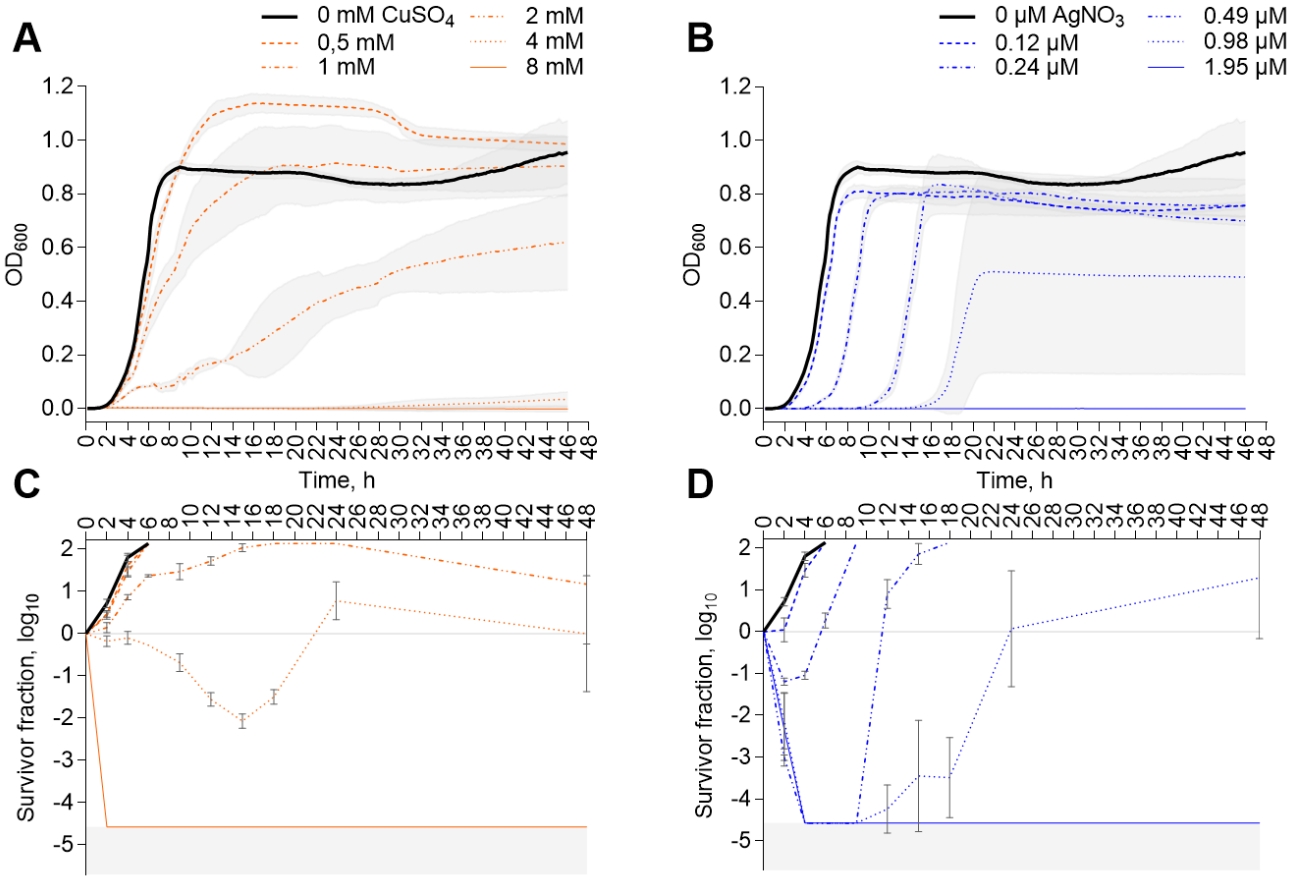

Figure 1. Growth (A, B) and kill (C, D) kinetics of *Escherichia coli* BW25113 exposed to $CuSO_4$ (A, C) or $AgNO_3$ (B, D) in the range of 0.0625-1 xMIC value of 8 mM Cu or 1.95µM Ag. Mean and SD of 3 (C, D) to 4 (A, B) biological replicates are shown. All growth curves from individual experiments represented on panels A and B can be found in Supplementary Fig S2 and numerical data in Supplementary Data file. Grey shading on A and B denotes standard deviation and on C and D value range below post-exposure colony counting detection limit with no colonies detected.

Upon closer examination of viable cell counts during the lag phase, a distinct difference in kill kinetics emerged: copper did not decrease viable counts except for the highest sub-MIC concentration of 4 mM concentration (0.5 xMIC) with long lag and severely inhibited growth (Fig. 1A, 1C), whereas silver caused rapid reduction in viable counts prior to resumption of normal exponential growth (Fig. 1D). Up to over 4-log reduction in viable counts underlies the seemingly longer lag times due to lower viable cell count at the onset of exponential growth consistent with the dilution effect on lag duration. This strong negative correlation between inoculum density and lag phase duration was also evident in non-exposed *E. coli* (Supplementary Fig S1 and S3). Prolonged physiological lag time can indicate responses to stressors (*11*), but when such changes occur in a dose-dependent manner in response to antimicrobial agents, sub-MIC killing and not

changes in single cell physiology may underlie the observed increase in lag time at population level.

In the light of these results, repeated or prolonged sub-MIC copper exposures rather select for resistance and silver exposures for tolerance. Tolerance—survival without growth—is easier to develop than resistance, which requires growth in the presence of an antimicrobial agent. Tolerance is thus considered a precursor to resistance(*9*, *10*), offering survivors time to acquire adaptive traits. In case of copper, selective advantage should grant a benefit overcoming the continuous growth inhibition. In contrast, during silver exposure, bacteria only need to survive until the silver is detoxified by biotic or abiotic factors in the environment, allowing the more tolerant fraction of the phenotypically heterogenous population to resume normal exponential growth. These findings highlight important differences for repeated antimicrobial use.

While both silver and copper are used in commercial antimicrobial products (*e.g.* wound care, textiles, touch surface materials) copper resistance is more studied in the context of environmental pollution or colonization of the indoor environment. Silver resistance, on the other hand, is a well-known challenge in *e.g.* burn wound care (*12*, *13*), necessitating combined treatment strategies. This raises the question of whether silver-based antimicrobial applications generally pose a higher risk for the development of tolerance and resistance compared to copper-based applications, due to the distinct inhibition and kill kinetics of copper and silver demonstrated by the current study. It also prompts further investigation into how these differences influence the co-selection of antibiotic tolerance and development of antibiotic resistance in medical applications.

Methods

Escherichia coli K-12 derivative BW25113 was used for the current study as a well-described model strain of *E. coli*. Lysogeny broth (LB: 5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl with optional 15 g/L bacteriological agar) and 37°C incubation with or without 150 rpm orbital shaking was used for all precultures and phosphate-buffered saline (PBS: 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) for washing and serial dilutions of cultures. Metal exposures used MOPS Minimal Medium (MMM)(*14*, *15*) with final glucose concentration of 0.4% with 0.4% glucose, 0.4% casamino acids, and 20 µg/L tryptophan. Further details on strain, media and growth conditions can be found in Supplementary Note 1.

Second subculture of *E. coli* on LB was used to inoculate overnight liquid LB cultures. The stationary culture was 100-fold diluted into fresh LB and grown to exponential phase (OD_{600}=0.5-0.7), then washed twice with cold PBS (4°C, 4400 g, 10 min). Pellets were resuspended in 2× MMM and adjusted to OD_{600} = 0.002 (~10^6 CFU/mL). Inoculum was added to twofold dilution series of $AgNO_3$ or $CuSO_4$or mixed 1:1 with deionized water and serially diluted in 1× MMM to test inoculum effects. The final volume of exposures in 96-well suspension culture plates was 150 µl for growth curves and 200 µl for kill curves. Sterile water was pipetted between the wells and plates sealed with parafilm to reduce drying during long incubations.

Growth curves were measured at OD_{600} every 15 min at 37°C with double-orbital mixing (BioTek Synergy H1, Agilent). Blank-corrected data were analyzed with Dashing Growth Curves at https://dashing-growth-curves.ethz.ch/ (*16*).

For kill curves, exposure plates were incubated at 37°C, 150 rpm in the dark. At each point, three concentration series per metal were serially diluted in PBS and drop-plated on LB; 150 µL of undiluted exposures were also spread-plated on 10 cm LB plates. Survival was expressed as log-transformed survivor fraction (post-/pre-exposure viable counts).

Statistical analyses were done in GraphPad Prism 10.4.1 using one-way ANOVA with post-hoc tests for multiple comparisons ($\alpha = 0.05$) across 3–4 biological replicates, as detailed in figure legends.

Acknowledgements

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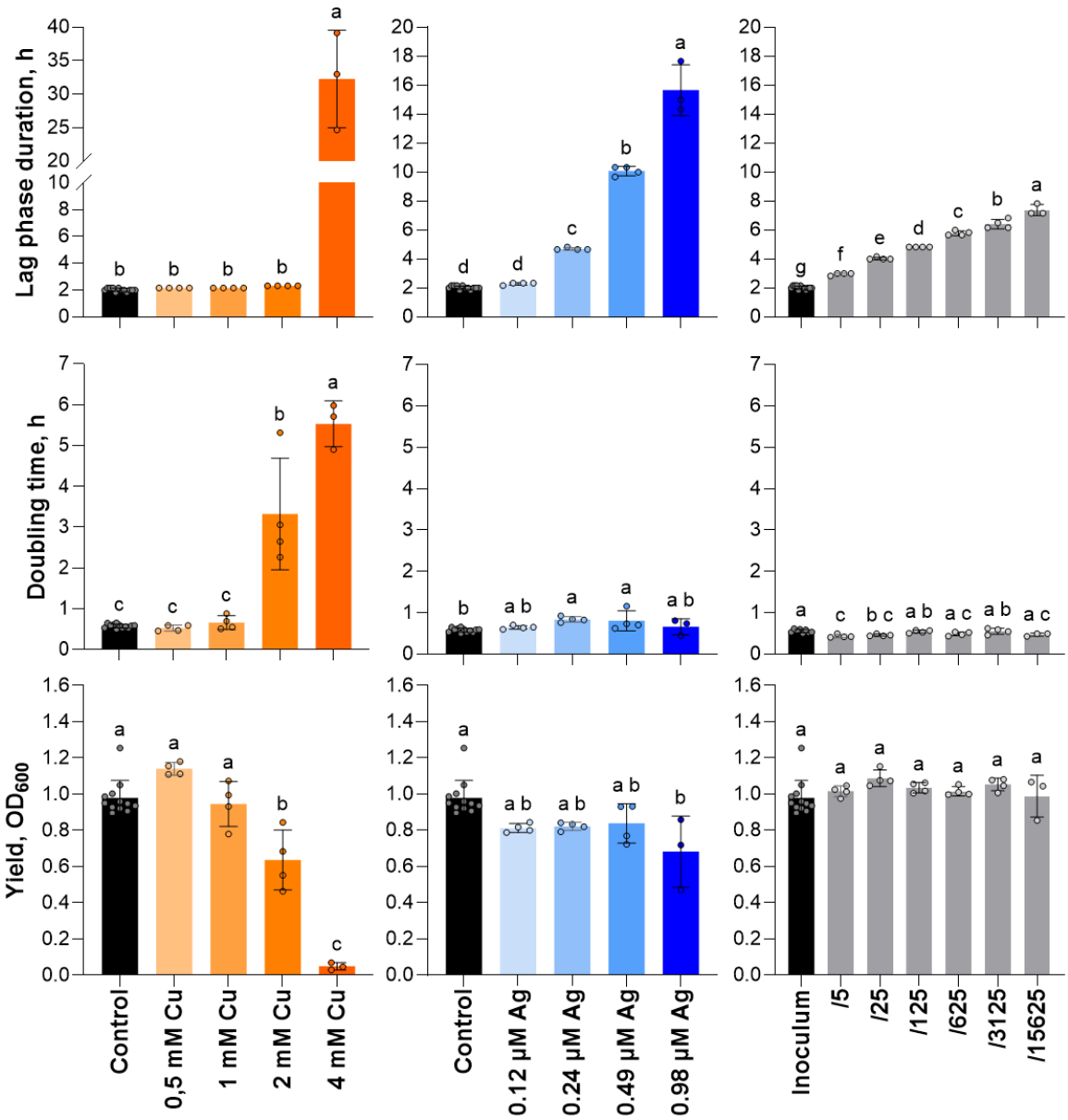
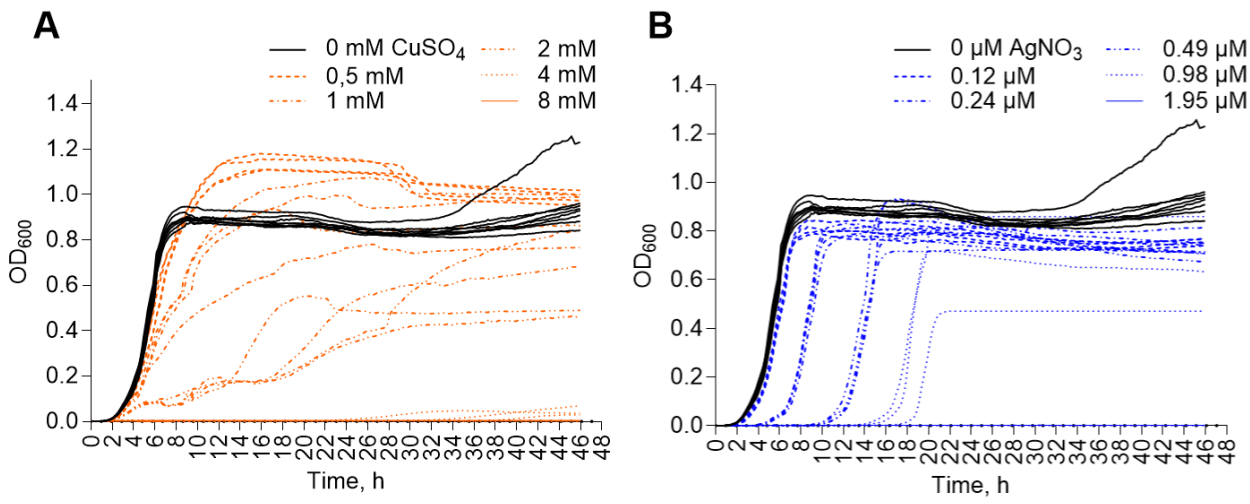
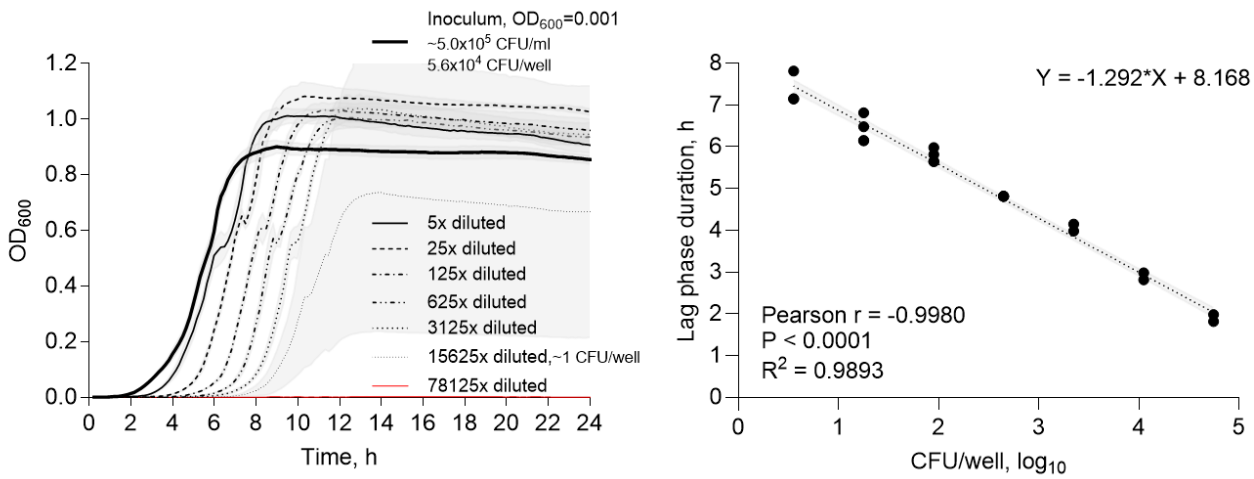
Supplementary Figures and Notes



Supplementary Figure S1. Growth parameters of *E. coli* BW25113 exposed to $CuSO_4$ (orange) or $AgNO_3$ (blue) or inoculums with different viable cell densities (grey). Values are calculated from growth curves on Supplementary Figure S3. Nominal metal concentration or inoculum fold dilution is marked on X-axis where appropriate. Single data points with mean and SD are shown. Lower case letters denote similarity groups based on one-way ANOVA followed by Tukey post-hoc test for multiple comparisons at α=0.05.



Supplementary Figure S2. Growth curves of *E. coli* BW25113 in the presence or absence of $CuSO_4$ (A) or $AgNO_3$ (B). Individual curves from 4 experiments underlying the mean curves on Figure 1 are shown. Non-exposed controls (black) on both panels are pooled across all experiments.



Supplementary Figure S3. Effect of inoculum density of *E. coli* BW25113 on growth kinetics (left) and negative correlation between the lag phase duration and inoculum density (right). Results from 4 biological replicates are presented.

Supplementary Note 1 on Strain selection, growth and exposure conditions.

Strain and inoculum selection

We first observed the effects described in this study for *E. coli* ATCC 8739 (unpublished data), a strain that is routinely used in antimicrobial testing. However, the strain tends to rapidly acquire silver resistance in liquid medium granted by mutations in the cryptic genomic *sil* locus [i,ii,iii] that interferes with studying the effects of antimicrobial metals on the wild-type strain. *E. coli* K-12 derivative BW25113 [iv] was used for the current study as a better described model strain of *E. coli* lacking the *sil* locus. Exponential phase inoculum was used in the study similarly to standardized MIC and MBC assays and to avoid potential biases caused by inoculating stationary or biofilm cultures into fresh growth-supporting exposure medium. Due to the limited growth phase coverage, the results cannot be generalized to stationary and colony-biofilm cultures in which cases additional experiments would be necessary.

Exposure medium selection

MOPS Minimal Medium (MMM) [v,vi] was selected for metal exposures due to its buffering capacity to reduce the acidification of growth medium upon addition of $CuSO_4$ as well as defined characteristics and lower organic content to control the metal ion speciation effects on bioavailability and thus toxicity to bacteria (*e.g.* formation of insoluble salts and complexing of metal ions by thiol groups). Both silver and copper ions can be complexed by organic contents, especially cysteine residues, of the medium with variable affinity, while only silver forms practically insoluble AgCl in the presence of 61 mM chloride ions in MMM. However, similar situations would be encountered in clinically relevant conditions. Chloride content of MMM is lower than in LB (171 mM in Miller and 86 mM in Lennox formulations), PBS (140 mM) or human blood plasma (~100 mM).

Re-growth conditions

We have previously observed that antimicrobial metal exposure can cause slower colony growth substantially affecting results of agar MIC assays [vii]. Therefore, growth and kill kinetics were recorded for 48 h instead of 24 h and all viable count platings from the time-kill experiments were checked for delayed growth. Drop-plated colonies from the kill curve experiments were counted after 14-16 h for optimal countable colony size and re-checked after 24, 48 and 72 h. Spread-plated colonies were counted after 24 h and checked 48 and 72 h for possible slower growth from exposures with higher metal concentration. No delayed emergence of colonies was observed. Exposure plates from the growth and kill kinetics experiments were kept at 37°C and 150 rpm for 5 days after the 48-h data collection and visually confirmed that delayed growth (emergence of visible turbidity) was not observed for metal concentrations at which no OD increase had been detected.

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