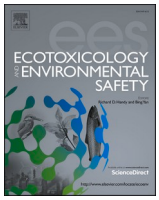
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Heavy metals promote the formation of multidrug-tolerant *Staphylococcus aureus* and *Escherichia coli* persisters

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ABSTRACT

Bacterial persisters are dormant phenotypic variants that are tolerant to antibiotics, contributing to treatment failure and the emergence of antimicrobial resistance. Although the formation of persisters has been extensively studied in regards to bacterial infections and treatment, such as antibiotic exposure or intracellular survival within macrophages, the role of environmental stressors in persister formation remains largely unexplored. In this study, we investigate the role of environmental heavy metals, specifically arsenic (As), cadmium (Cd), and mercury (Hg), in promoting persister cell formation in *Staphylococcus aureus* and *Escherichia coli*. Log-phase cultures were exposed to heavy metals (5 mM As, 1.25 mM Cd, 4 μM Hg for *S. aureus*; 12.5 mM As, 2 mM Cd, and 15 μM Hg for *E. coli*) for 0.5 h to induce persister cells. We observed that exposure to these metals induced persister cell formation, confirmed by intracellular ATP levels through microscopy and luciferase assays, as well as by reactive oxygen species (ROS) levels using carboxy-H2DCFDA. Short-term heavy metal exposure strongly depleted intracellular ATP while generating ROS. Moreover, we observed enhanced expression of genes involved in the SOS response, including *recA*, *umuC*, *dinB*, *rexA*, *rexB*, *sulA*, *rpoS*, and *soxR*, as measured by qPCR. This response was likely induced by elevated ROS levels following heavy metal exposure. Furthermore, we demonstrate that heavy metal-induced bacterial persisters exhibited a substantially increased emergence of antibiotic resistance, as shown by ciprofloxacin resistance developing in the presence of heavy metals. Therefore, our results clearly demonstrate that heavy metals can induce persister cells by depleting cellular ATP and generating ROS, and these bacterial responses to heavy metals substantially contribute to antibiotic resistance. These findings highlight the intricate relationship between environmental heavy metals, bacterial persister formation, and antibiotic resistance, emphasizing the need for a "One Health" strategy to address the growing antibiotic resistance crisis.

1. Introduction

Bacteria have been exposed to naturally occurring antimicrobial agents for millions of years (D'Costa et al., 2011; Wright, 2019). These agents, produced by other microbes such as fungi and bacteria, play important roles in microbial competition for resources. This prolonged evolutionary exposure has allowed bacteria to develop survival strategies, enabling them to tolerate lethal concentrations of antimicrobial agents (Bhullar et al., 2012; D'Costa et al., 2011; Waglechner et al., 2021). With the advent of semisynthetic and novel antibiotics and their extensive use by humans, these evolutionary processes have accelerated, strengthening bacterial defenses (Walsh et al., 2023). One of the most well-established survival strategies is antibiotic resistance, which bacteria acquire through *de novo* mutation or horizontal gene transfer (Windels et al., 2020). Antibiotic resistance mechanisms include degradation of antimicrobial agents, target modification, and efflux pumping of antibiotics, which effectively mitigate antibiotic potency (Darby et al., 2023). Antibiotic resistance is heritable, allowing progeny to survive and proliferate under lethal antibiotic stress. Additionally, resistance can spread across environments, animals, and humans,

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contributing to global rise in antibiotic-resistant pathogens (Liu et al., 2016; Pruden et al., 2012; Zhang et al., 2022). This interconnected spread elevates antimicrobial resistance to a critical "One Health" concern, which recognizes the interdependence of human, animal, and environmental health (Hernando-Amado et al., 2019; McEwen and Collignon, 2018).

Beyond antibiotic resistance, bacteria employ another strategy to survive lethal antimicrobial pressures by forming a subpopulation called persisters. Cells in this population are dormant, nondividing bacterial cells that tolerate antibiotics without genetic resistance. Unlike resistant bacteria, which grow in the presence of antibiotics, persisters survive by entering a temporary, metabolically silent state. This small fraction of cells persists under antibiotic treatment (Balaban et al., 2019a; Lewis, 2010). Most antibiotics target essential biosynthetic processes that are activated during bacterial growth (Lewis, 2010), with some relying on metabolic activity for uptake (Becker and Cooper, 2013). Therefore, the nongrowing, metabolically dormant state of persisters leads to the inactivation of antibiotic targets or prevents antibiotic uptake, leading to their survival under attack. This phenomenon, termed as antibiotic tolerance, arises not from genetic mutations but from stochastic processes that arrest growth or lead to metabolic dormancy (Balaban et al., 2019b). Unlike antibiotic resistance, this tolerance mechanism acts against multiple antibiotics with different targets (Balaban et al., 2019b; Lewis, 2010). Bacterial persisters function as antibiotic resistance development reservoirs as they survive antibiotic treatments and encourage the emergence and selection of resistant mutants during subsequent regrowth phases (Windels et al., 2019). Antibiotic-tolerant persisters are clinically significant because they are responsible for biofilm-related and chronic infections, for which effective antibiotic options are limited (Ciofu et al., 2022; Fisher et al., 2017; Gollan et al., 2019).

Persister formation has been investigated in the context of bacterial infections and treatment. Paradoxically, antimicrobial agents are known to induce persister formation. For example, bacteriostatic antibiotics, such as rifampin, chloramphenicol, and tetracycline, promote persister formation by inhibiting mRNA or protein synthesis, ultimately leading to a quiescent state (Kwan et al., 2013; Niu et al., 2023; Ocampo et al., 2014). Carbonyl cyanide m-chlorophenylhydrazone, which is an antimicrobial ionophore, promotes persister formation by dissipating proton motive force (PMF), leading to inactivation of bacterial ATPases, and subsequently reducing ATP production (Kwan et al., 2013; Pu et al., 2019; Strahl and Hamoen, 2010; Wang et al., 2021). Furthermore, intracellular stressors encountered within host macrophages, such as reactive oxygen species (ROS), reactive nitrogen species, acidic pH, or nutrient deprivation, drive persister formation by inhibiting the tricarboxylic acid (TCA) cycle, cellular respiration, and ATP production as well as by activating toxin–antitoxin modules or triggering the SOS response (Helaine et al., 2014; Ronneau et al., 2023; Rowe et al., 2020). Collectively, these findings illustrate that multiple stimuli can drive the generation of antibiotic-tolerant persisters.

Heavy metals, such as lead, mercury, cadmium, vanadium, and arsenic, have high atomic weights and densities, typically greater than those of water. Known for their environmental toxicity, persistence, and bioaccumulation, heavy metals pose a growing threat due to increasing levels of anthropogenic activities (Ahmad et al., 2021; Ali et al., 2019). Furthermore, heavy metals can gravely affect bacteria through several mechanisms, including DNA damage, protein destruction, and membrane disruption (Igiri et al., 2018; Li et al., 2021; Zhang et al., 2023). These stressors not only hinder bacterial survival but also create selective pressures that can induce the development and spread of antimicrobial resistance. Resistance genes for heavy metals and antibiotics are often colocated on mobile genetic elements, enabling coselection and enhancing resistance to heavy metals as well as antibiotic agents (Li et al., 2019; Mazhar et al., 2021; Thomas et al., 2020; Zhong et al., 2021). However, the effects of heavy metals on persister formation remain largely unexplored.

In this study, we present compelling evidence that heavy metals promote bacterial persister formation with high tolerance to various bactericidal antibiotics. Our findings demonstrate that exposure to heavy metals leads to bacterial cellular ATP depletion, PMF dissipation, and intracellular ROS accumulation, along with altered expression of SOS response-related genes, collectively triggering persister formation. Moreover, persister cells induced by heavy metal exposure exhibit elevated potential for antibiotic resistance development. These results underscore the pivotal role of environmental heavy metals in promoting antibiotic-tolerant persisters and facilitating emergence of antibiotic-resistant bacteria, posing a serious threat to ecosystems and public health. Therefore, our results show that heavy metals can drive persister cell formation by depleting cellular ATP and generating ROS, and these bacterial responses substantially contribute to the development of antibiotic resistance, highlighting the need for a One health approach to address the interconnected challenges of environmental pollution and antibiotic resistance.

2. Materials and methods

2.1. Bacterial strains and chemical compounds

Staphylococcus aureus MW2 and *Escherichia coli* MG1655 were used for this study. The *S. aureus* MW2 strain was cultured in cation-adjusted Muller–Hinton broth (CaMH; BD Difco, Franklin Lakes, NJ) or on CaMH agar, and *E. coli* MG1655 was cultured in Lennox broth (LB; BD Difco) or on LB agar. Bacteria were routinely grown at 37 °C with shaking at 200 rpm. The antibiotics, which included gentamicin (Sigma-Aldrich, Burlington, MA), ciprofloxacin (Sigma-Aldrich), ofloxacin (Sigma-Aldrich), cefotaxime (Sigma-Aldrich), and vancomycin (Sigma-Aldrich), were prepared as stock solutions at concentrations of 10 mg/mL; they were diluted to appropriate concentrations before use. The heavy metals in the form of sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$; Sigma-Aldrich), cadmium chloride ($CdCl_2$; Sigma-Aldrich), and mercuric chloride ($HgCl_2$; Sigma-Aldrich) were dissolved in double distilled water to stock concentrations of 1 M or 1 mM; they were further diluted before use.

2.2. Induction of bacterial persister cells

Overnight bacterial cultures were re-inoculated at a ratio of 1:10,000 in 25 mL of appropriate media in a 250 mL Erlenmeyer flask and then incubated at 37 °C with shaking at 200 rpm. The inoculated cultures were grown for 2–3.5 h until they reached an OD_{600} of approximately 0.2–0.3. Subsequently, heavy metals were added to the cultures at persistence-inducing concentrations of 4 × the minimum inhibitory concentration (MIC), as follows: 5 mM arsenate, 1.25 mM cadmium, and 4 μM mercury for *S. aureus* MW2 and 12.5 mM arsenate, 2 mM cadmium, and 15 μM mercury for *E. coli* MG1655. The cultures were then incubated for an additional 30 min with shaking at 200 rpm. After incubation, heavy metal-induced persister cells were washed three times with phosphate-buffered saline (PBS), harvested, and prepared for the subsequent analyses.

2.3. Antibiotic tolerance assays

Antibiotic tolerance was assessed as described previously (Allison et al., 2011; Conlon et al., 2013; Kim et al., 2019). Briefly, heavy metal-induced bacterial persister cells were challenged with 100 × MIC of the respective antibiotics (50 μg/mL gentamicin, 25 μg/mL ciprofloxacin, and 100 μg/mL vancomycin for *S. aureus* MW2 and 100 μg/mL gentamicin, 20 μg/mL ofloxacin, and 10 μg/mL cefotaxime for *E. coli* MG1655) for 4 h. At specified time points, samples were collected, washed with PBS, and plated onto appropriate agar. After overnight incubation at 37 °C, the viability was determined by colony enumeration.

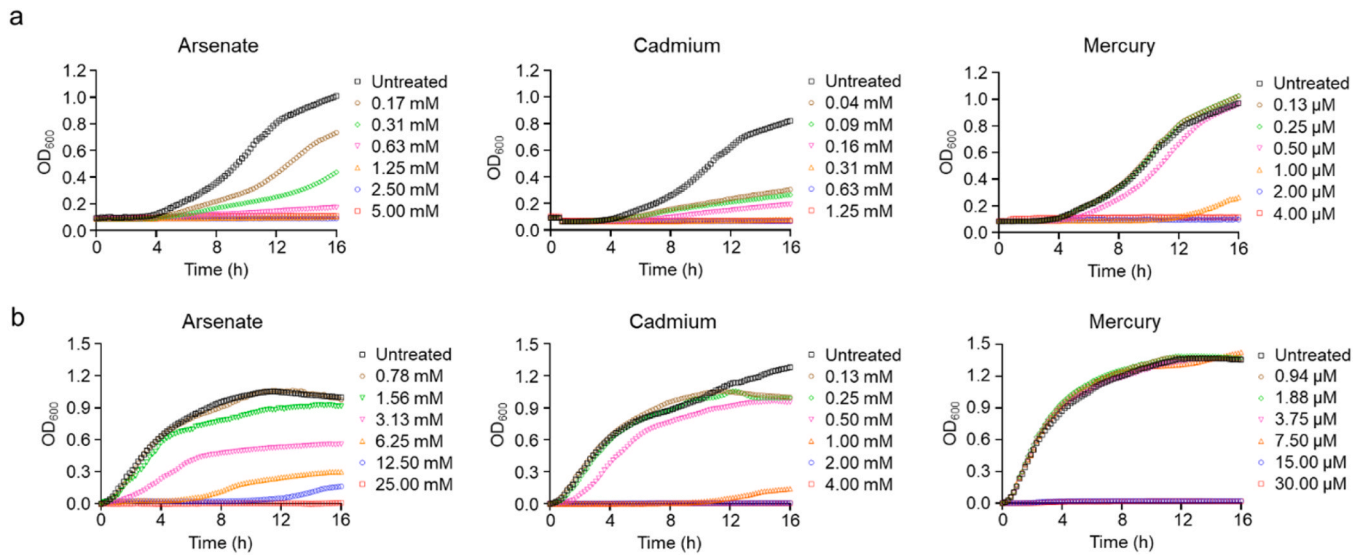


Fig. 1. Heavy metals induced growth arrest. *S. aureus* MW2 **(a)** and *E. coli* MG1655 **(b)** were treated with the indicated concentrations of arsenate, cadmium, and mercury, and bacterial growth was monitored over 18 h by measuring the absorbance at 600 nm every 10 min using a plate reader. Culture media without heavy metals were used as an untreated control (Untreated). Results are presented as the mean of three biological replicates (n = 3); error bars are omitted for clarity.

2.4. Measurement of intracellular ATP levels

Intracellular ATP levels were measured using a BacTiter-Glo Microbial Cell Viability Assay Kit (Promega, Madison, WI) as described previously (Heo et al., 2024). The reagent mixture and buffer were prepared according to the manufacturer's instructions. After treating exponentially growing bacterial cells with arsenate, cadmium, or mercury, cells were washed three times with PBS and resuspended in PBS to an OD_{600} of 0.2. Subsequently, 100 μL aliquots of the cell suspension were dispensed into each well of a black, clear-bottom 96-well plate (Corning, Corning, NY), followed by the addition of 100 μL of the reagent mixture. Luminescence was then measured using Cytation 5 multimode plate reader (BioTek, Winooski, VT).

2.5. Fluorescence microscopy

For fluorescence microscopy analysis, we prepared a fluorescent reporter strain of MW2 harboring plasmids P*cap5A*::*gfp* and P*arcA*::*gfp*

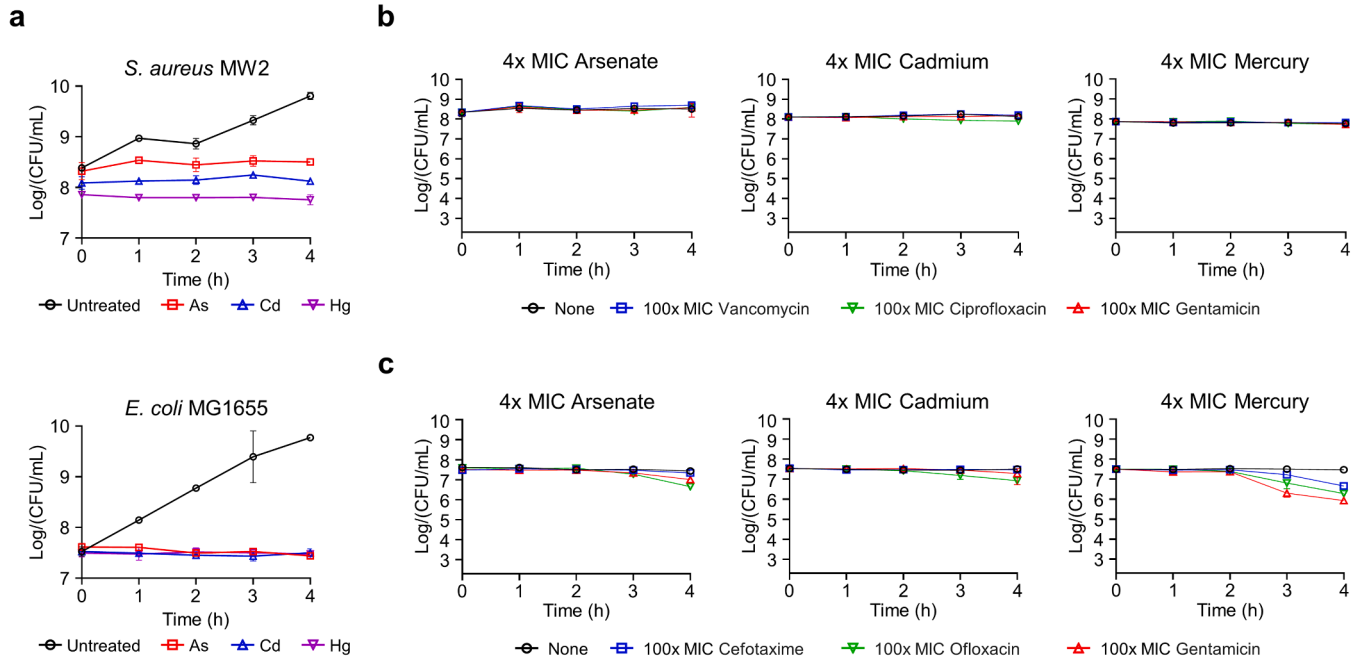


Fig. 2. Heavy metals induced the formation of antibiotic-tolerant persister cells. (a) Exponentially growing *S. aureus* MW2 and *E. coli* MG1655 were exposed to 4 × MIC of the indicated heavy metals for 4 h, and the bacterial viability was assessed at hourly intervals. The group without heavy metals was used as untreated control (Untreated). **(b, c)** *S. aureus* MW2 and *E. coli* MG1655 were treated with the indicated heavy metals for 30 min. The heavy metal-treated *S. aureus* **(b)** and *E. coli* **(c)** were subsequently challenged with 100 × MIC of the indicated antibiotics for 4 h. Viable cell counts were determined by serial dilution and plating on CaMH agar plates. The group with no antibiotic treatment was used as a positive control (None). Error bars represent the standard deviation (SD) of three biological replicates.

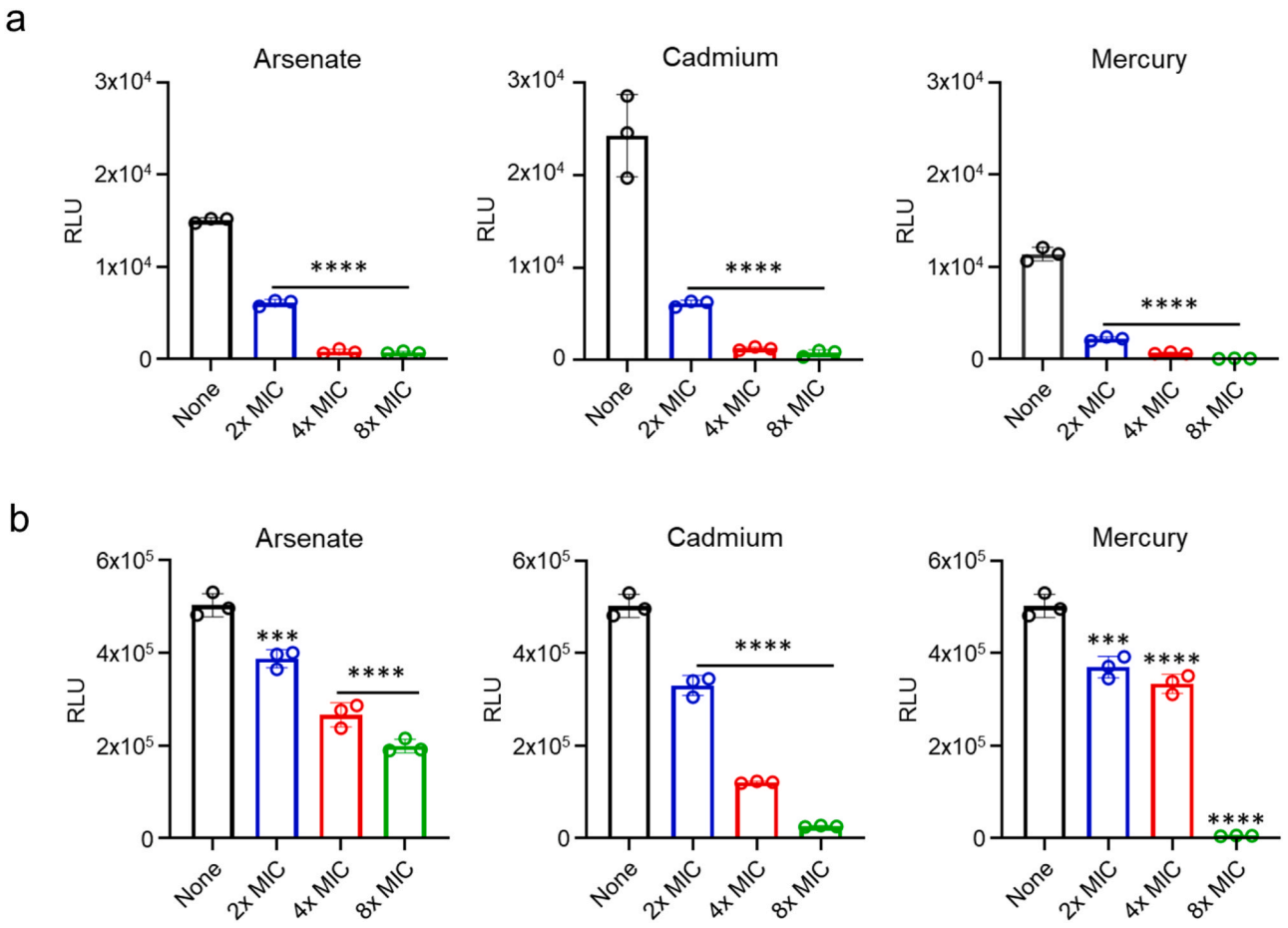


Fig. 3. Heavy metals induced ATP depletion in bacteria. Exponentially growing *S. aureus* MW2 **(a)** and *E. coli* MG1655 **(b)** were treated with 0 × , 2 × , 4 × , and 8 × MIC of the indicated heavy metals for 30 min. The group with no antibiotic treatment was used as a positive control (None). The intracellular ATP levels were measured using a bioluminescent luciferin luciferase assay. Data points represent individual measurements, with error bars showing the mean ± SD (n = 3). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test (***p < 0.001, ****p < 0.0001).

(Conlon et al., 2016). In brief, the plasmids were electroporated into *S. aureus* RN4220 and transduced to *S. aureus* MW2 using a φ11 lysate. To prepare the samples for fluorescence imaging, overnight cultures of *S. aureus* MW2 were diluted 1:10,000 and re-inoculated into tryptic soy broth and grown until an OD_{600} of approximately 0.2 was achieved. Subsequently, the cultures were treated with heavy metals as indicated earlier, followed by incubation for 30 min at 37 °C. The overnight cultures were used as the stationary phase, whereas the log-phase cultures were used as the untreated control. After incubation, the cells were washed three times with PBS and concentrated 10-fold. Image acquisition was performed on an agarose gel pad at room temperature using a Leica DMi8 M inverted microscope with a × 100 oil immersion objective lens (Leica Microsystems, Wetzlar, Germany).

2.6. Assessment of PMF dissipation

PMF disruption was evaluated using the fluorogenic probe 3,3′-dipropylthiadicarbocyanine iodide, $DiSC_3(5)$ (Sigma-Aldrich, St. Louis, MO) (Kim et al., 2024). *S. aureus* MW2 and *E. coli* MG1655 were grown to the exponential phase in CaMH broth and resuspended in 0.1 M potassium phosphate buffer containing 0.2 % glucose and 1 % DMSO until an OD_{600} of 0.2 was achieved. Subsequently, 50 μL aliquots of the cell suspension were added to each well of a black, clear-bottom 96-well plate (Corning). $DiSC_3(5)$ was prepared to a final concentration of 4 μM from a 1-mM stock solution in the same buffer and added to the wells at a 1:1 ratio. Fluorescence measurements were recorded for 30 min using Cytation 5 multimode plate reader (BioTek) with excitation set at 610 nm and emission set at 660 nm until the fluorescence intensity stabilized to the baseline. After stabilization, 100 μL of the heavy metal solutions, prepared at the indicated concentrations, was added to each well and measurements were continued at the same wavelengths for another 30 min.

2.7. Evaluation of intracellular ROS levels

Intracellular ROS generation in *S. aureus* MW2 and *E. coli* MG1655 after exposure to heavy metals was assessed using the fluorescent dye carboxy-H_2DCFDA (Thermo Fisher, Waltham, MA), which is a general indicator of oxidative stress (Rutherford et al., 2010). *S. aureus* MW2 and *E. coli* MG1655 were cultured in CaMH broth to an OD_{600} of 0.2 for *S. aureus* MW2 and an OD_{600} of 0.3 for *E. coli* MG1655. Furthermore, carboxy-H_2DCFDA was added to each culture at a final concentration of 10 μM, after which 50 μL aliquots of the cell and dye mixtures were dispensed into each well of a black 96-well plate (Corning) containing the indicated concentrations of heavy metals. Fluorescence measurements were conducted at 37 °C for 2 h using Cytation 5 multimode plate reader (BioTek), with the excitation and emission wavelengths set to 495 nm and 520 nm, respectively. The OD_{600} values were simultaneously measured to account for cell density, and fluorescence readings were normalized to OD_{600}.

2.8. Measurement of expression of SOS system-related genes

Total RNA of the bacterial persisters was isolated using an RNeasy

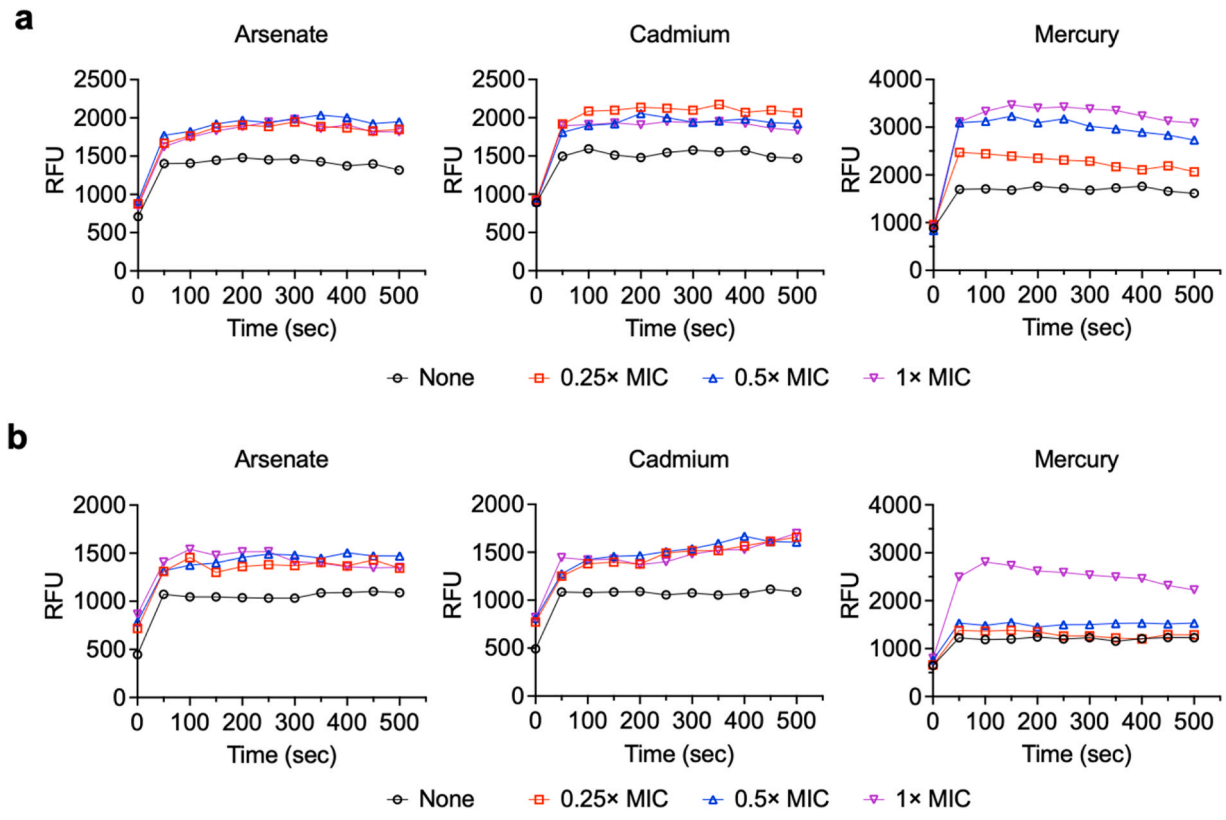


Fig. 4. Heavy metals dissipated the proton motive force. Exponentially growing *S. aureus* MW2 **(a)** and *E. coli* MG1655 **(b)** were treated with 0, 0.25 × , 0.5 × , and 1 × MIC of the indicated heavy metals. The group with no antibiotic treatment was used as a positive control (None). The proton motive force was monitored by measuring the fluorescence of the $DiSC_3(5)$ dye at an excitation wavelength of 610 nm and an emission wavelength of 660 nm after heavy metal treatment. Results are shown for one of three independent biological triplicates for clarity.

Mini Kit (QIAGEN, Valencia, CA), and 5 μg of total extracted RNA was synthesized into cDNA using RNA to cDNA EcoDry Random Hexamer Premix (Takara, Japan). Furthermore, quantitative real-time polymerase chain reaction (PCR) was conducted with EzAmp™ qPCR 2X Master Mix (Elpis Biotech, Korea) according to the manufacturer's instructions. The qRT-PCR reaction mixture was amplified on a StepOnePlus™ real-time PCR system (Applied Biosystems, Foster City, CA) at a condition of 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C.

2.9. Antibiotic resistance development assay

Antibiotic resistance development assay was performed through a 14-day serial passage (Heo et al., 2024). Ciprofloxacin was prepared by performing 2-fold serial dilution in CaMH broth, starting from initial concentrations of 40, 48, and 56 μg/mL, distributed across three columns of a 96-well plate, resulting in total of 24 ciprofloxacin concentrations. Next, 50 μL of heavy metal-induced bacterial persister cells (OD_{600} of ~0.005 in CaMH) was inoculated in each well of the 96-well plate containing ciprofloxacin. Bacterial culture not exposed to heavy metals was used as an untreated control. The plates were incubated at 37 °C for 24 h, after which the optical density at 600 nm was measured using Cytation 5 multimode reader (BioTek, USA). Bacterial growth was defined at an OD_{600} of ≥ 0.1. Cultures from a sub-MIC exhibiting growth were diluted 1,000-fold in 2 mL of CaMH in a 15-mL test tube, and cultured until they reached an OD_{600} of 0.2. These cultures were then treated with the indicated concentrations of heavy metals for 30 min. After treatment, the samples were transferred to a fresh ciprofloxacin gradient plate for the next passage. The remainder of the sample was mixed with glycerol to a final glycerol concentration of 16 % and stored at −80 °C.

3. Results

3.1. Heavy metals induce growth arrest

Bacterial persisters are characterized by nongrowing dormant states, prompting us to investigate the effects of environmental heavy metals on bacterial growth. Herein, we used the following two well-established bacterial strains known for persister formation: gram-positive methicillin-resistant *S. aureus* MW2 and gram-negative *E. coli* MG1655 (Kim et al., 2018; Shan et al., 2017). We exposed these two strains to varying concentrations of three environmentally important heavy metals (arsenic, cadmium, and mercury) for 16 h. As these heavy metals exist naturally in ionic forms such as AsO_4^{3-}, Cd^{2+}, and Hg^{2+} (Choong et al., 2014; Cubadda et al., 2017; Liu et al., 2017), we used sodium arsenate, cadmium chloride, and mercuric chloride for our experiments.

As illustrated in Fig. 1, exposure to all tested heavy metals resulted in bacterial growth inhibition. Specifically, *S. aureus* MW2 exhibited complete growth arrest when treated with 1.25 mM arsenate, 0.312 mM cadmium, or 2 μM mercury (Fig. 1a). Similarly, *E. coli* MG1655 showed substantial growth inhibition when treated with 25 mM arsenate, 4 mM cadmium, or 15 μM mercury (Fig. 1b). The inhibition patterns varied, with some exhibiting gradual dose–response inhibition and others exhibiting sharp inhibition beyond a certain concentration threshold (Fig. 1). These patterns are consistent with those observed in antibiotic treatments, where gradual inhibition is observed with oxacillin, and sharp inhibition occurs with ciprofloxacin, gentamicin, and vancomycin (Fig. S1, Table S1). These results suggested that the inhibitory pattern may be related to bacterial sensitivity to specific agents.

To quantify the inhibitory activity, the MICs of these metals were determined. Arsenate, cadmium, and mercury had MICs of 1.25 mM, 0.313 mM, and 1.25 μM, respectively, for *S. aureus* MW2, and

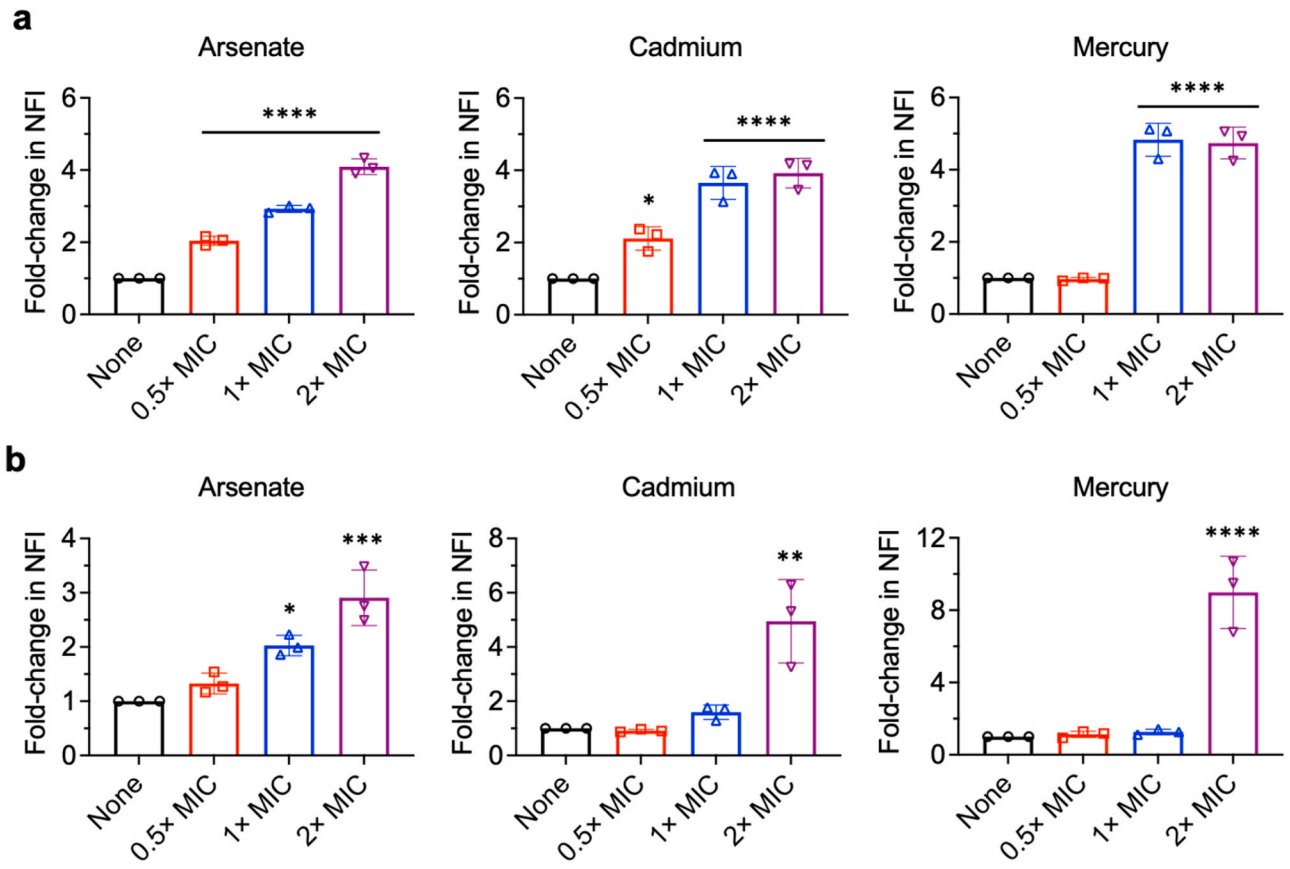


Fig. 5. Heavy metals triggered intracellular ROS accumulation. Exponential-phase cells of *S. aureus* MW2 **(a)** and *E. coli* MG1655 **(b)** were treated with 0, 0.5 ×, 1 ×, and 2 × MIC of arsenate, cadmium, and mercury for 2 h. The intracellular ROS levels in the heavy metal-treated cells were determined using a fluorescence probe carboxy-H2DCFDA. NFI (normalized fluorescence intensity) was calculated as the fluorescence intensity at excitation 495 nm and emission 520 nm, divided by the cellular optical density at 600 nm. The group with no antibiotic treatment was used as a positive control (None). All experiments were performed in triplicate, with individual data points shown; data are presented as the mean ± SD (n = 3). Statistical differences were analyzed using one-way ANOVA followed by Tukey's post hoc test (* $p < 0.5$, ** $p < 0.01$, and **** $p < 0.0001$).

3.125 mM, 0.5 mM, and 3.75 μM, respectively, for *E. coli* MG1655 (Table S1). Notably, mercury exhibited an MIC approximately one order of magnitude lower than arsenate and cadmium for both bacterial species (Table S1), indicating that *S. aureus* and *E. coli* are more sensitive to mercury than the other two heavy metals.

Additionally, we assessed whether these heavy metals could inhibit the growth of exponentially growing bacterial cells. As depicted in Fig. 2a, a 30-min treatment with arsenate, cadmium, or mercury at a level of 4 × MIC completely halted the growth of exponentially growing *S. aureus* and *E. coli* cells. Overall, these results demonstrate that arsenic, cadmium, and mercury can potently arrest bacterial growth at certain concentrations.

3.2. Heavy metals induce the formation of multidrug-tolerant bacterial persisters

We investigated whether the growth-arrested cells induced by heavy metal treatment exhibited antibiotic tolerance, which is a key phenotypic characteristic of bacterial persisters. Thus, we treated the heavy metal-arrested cells with 100 × the MIC of bactericidal antibiotics targeting different cellular processes: the cell wall synthesis inhibitors vancomycin and cefotaxime, the protein synthesis inhibitor gentamicin, and the DNA replication inhibitors ciprofloxacin and ofloxacin. Although all of the tested antibiotics rapidly killed the untreated bacterial cells (Fig. S2), heavy metal-treated *S. aureus* and *E. coli* cells showed no or only nominal decreases in viability over 4 h when exposed to 100 × MIC of the tested antibiotics (Fig. 2b, c). To exclude the possible generation of a resistant population due to heavy metal treatment, we also examined the MIC for each antibiotic and found that the MIC remained the same (Table S2). These results demonstrate that exposure to heavy metals promotes the transition of bacteria into persister cells, characterized by growth arrest and high tolerance to multiple antibiotics with different action mechanisms.

3.3. Heavy metals induce metabolic arrest and ROS accumulation

We explored the mechanism by which heavy metals promote bacterial persister formation. Since ATP depletion is known to induce persister formation (Conlon et al., 2016; Manuse et al., 2021), we first measured intracellular ATP levels. Consistently, exposure to arsenate, cadmium, or mercury resulted in a substantial reduction in intracellular ATP levels in *S. aureus* and *E. coli* compared to the untreated controls (Fig. 3a, b).

To further elucidate how these heavy metals reduce cellular ATP levels, we investigated their impact on PMF, which drives most ATP production in aerobic cultures. We used $DiSC_3(5)$, a fluorescent dye sensitive to PMF changes, whose fluorescence is quenched when the PMF is intact and increased as the PMF dissipates. As illustrated in Fig. 4, treatment with arsenate, cadmium, and mercury resulted in increased fluorescence in *S. aureus* and *E. coli*, indicating that these heavy metals rapidly dissipate PMF. This dissipation likely impairs the electron transport chain, resulting in subsequent ATP depletion.

Finally, we assessed whether heavy metals elevate levels of intracellular ROS, as ROS accumulation is known to promote persister

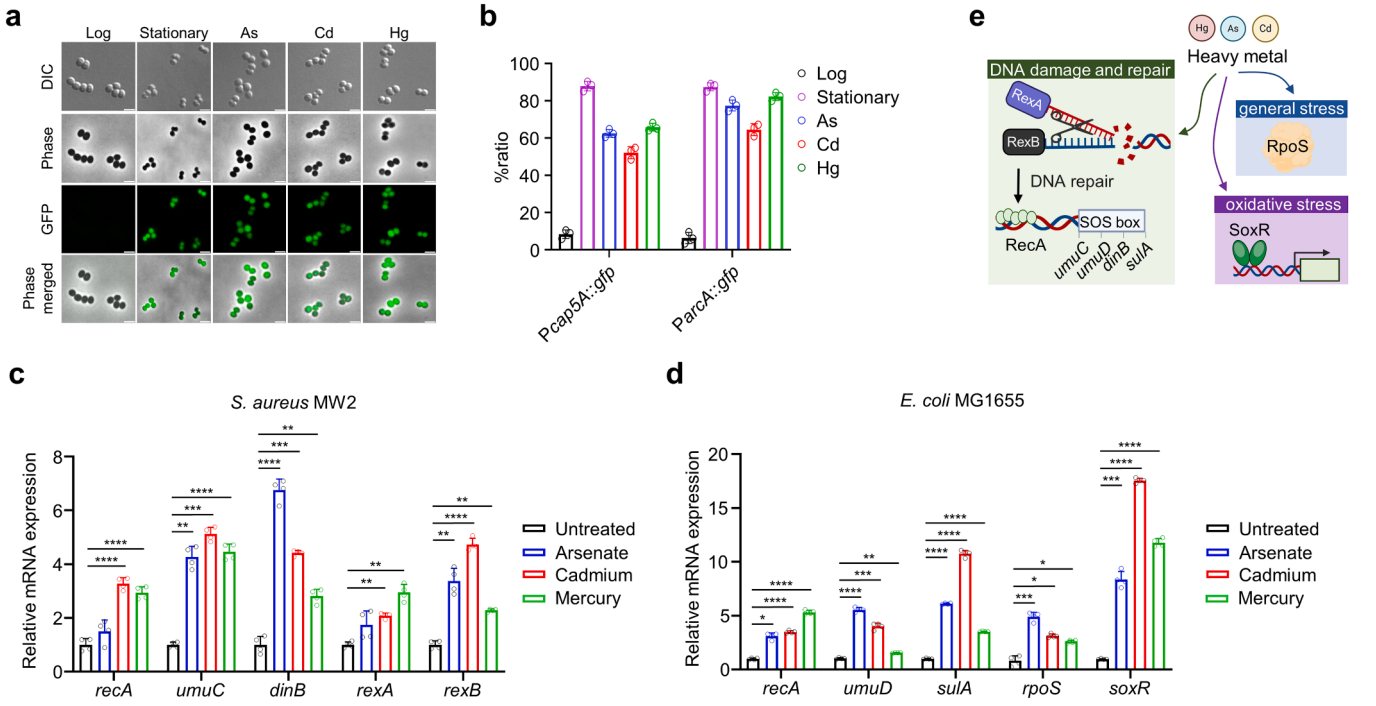


Fig. 6. Heavy metals induced persister formation indicator promoters and activated the SOS repair system. Exponentially growing *S. aureus* MW2 carrying plasmids P*cap5A*::*gfp* and P*arcA*::*gfp* were treated with 4 × MIC of arsenate (As), cadmium (Cd), and mercury (Hg) for 30 min. **(a)** Differential interference contrast, phase contrast (Phase), and fluorescence (GFP) microscopy images of *S. aureus* cells were obtained using a Leica DMi8 M inverted microscope with a × 100 oil immersion objective lens. **(b)** The percentage of the *S. aureus* cells exhibiting an GFP signal was quantified using Leica imaging software. Metal-untreated log-phase cells (Log) and the metal-untreated stationary-phase cells (Stationary) served as negative and positive controls, respectively. **(c, d)** Quantitative real-time PCR analysis of mRNA levels in *S. aureus* MW2 and *E. coli* MG1655 after treatment with heavy metals for 30 min. Relative gene expression was normalized to *gapA* expression. Data are presented as the mean ± SD (n = 4). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test with the following significance levels: *$p < 0.5$, * *$p < 0.01$, * **$p < 0.001$, * ** *$p < 0.0001$. (e) Schematic of heavy metal-induced stress response gene expression.

formation (Rowe et al., 2020; Wang et al., 2017; Wu et al., 2012). Arsenate, cadmium, or mercury treatment of *S. aureus* and *E. coli* cells resulted in significant intracellular ROS accumulation (Fig. 5). This buildup reflects an imbalance between ROS generation and bacterial scavenging defense systems, creating severe oxidative stress. The findings also indicated that heavy metals induce oxidative stress in bacteria, potentially disrupting key proteins involved in metabolic processes, such as the electron transport chain. This disruption likely leads to decreased ATP levels, thus promoting persister formation.

3.4. Heavy metals activate persister-indicator promoters and induce bacterial SOS repair system

Unlike *E. coli*, nearly all stationary-phase cells of *S. aureus* exhibit high antibiotic tolerance, which is a characteristic of persisters (Allison et al., 2011; Kim et al., 2018). In *S. aureus*, the promoter of the capsular polysaccharide operon (P*cap5A)* and the promoter of the arginine deiminase pathway (P*arcA*) are highly activated in stationary-phase persister cells, serving as reliable transcriptional indicators of this state (Conlon et al., 2016). To examine if heavy metals activate these persister-indicator promoters, we constructed *S. aureus* MW2 strains carrying P*cap5A and* P*arcA* promoters fused to green fluorescent protein (GFP), designated as *S. aureus* MW2 P*cap5A::gfp* and *S. aureus* MW2 P*arcA::gfp*, respectively. As expected, stationary-phase cells of *S. aureus* MW2 P*cap5A::gfp* and *S. aureus* MW2 P*arcA::gfp* exhibited a > 80 % increase in fluorescence compared to exponentially growing cells (Fig. 6a, b). Similarly, heavy metal-treated *S. aureus* cells showed a more than 50 % increase in fluorescence relative to the untreated controls, indicating that arsenic, cadmium, and mercury consistently activate these indicator promoters associated with persister formation.

ROS are stressors that cause DNA damage, triggering the bacterial SOS repair system (Podlesek and Bertok, 2020). To determine whether elevated ROS levels induced by heavy metal exposure could activate the SOS system, we determined the expression levels of key genes involved in this pathway, including those encoding SOS response regulators, error-prone polymerases, and DNA repair proteins. As depicted in Fig. 6c and 6d, nearly all tested genes in *S. aureus* MW2 and *E. coli* MG1655 were significantly upregulated after 30 min of heavy metal exposure. In *S. aureus*, *recA*, which encodes a key SOS response regulator, and *rexA*, a gene encoding a DNA repair protein, were particularly overexpressed following cadmium and mercury treatment, with a noticeable but not statistically significant, increase observed with arsenate treatment (Fig. 6c, d). Notably, *umuC* and *dinB*, which encode error-prone polymerases, and *rexB*, which encodes a DNA repair protein, were significantly overexpressed in *S. aureus* exposed to all three heavy metals. Similarly, in *E. coli*, genes including *recA*, *umuD*, which encode error-prone polymerases, *sulA*, which encodes a cell division inhibitor, *rpoS*, which encodes a sigma factor, and *soxR*, which encodes a superoxide response protein, were upregulated in response to arsenate, cadmium, or mercury exposure compared to the untreated controls (Fig. 6c, d). These findings demonstrate that ROS accumulation resulting from heavy metal exposure robustly activates the SOS system in bacteria (Fig. 6e).

3.5. Heavy metal-driven persisters confer a higher frequency of antibiotic resistance

Since the SOS response-triggered DNA repair machinery is known to promote the development of antibiotic resistance (Crane et al., 2021; Pribis et al., 2019), we hypothesized that persisters induced by heavy metal exposure could facilitate the acquisition of antibiotic resistance. To test this, we performed a 14-day serial passage experiment, exposing

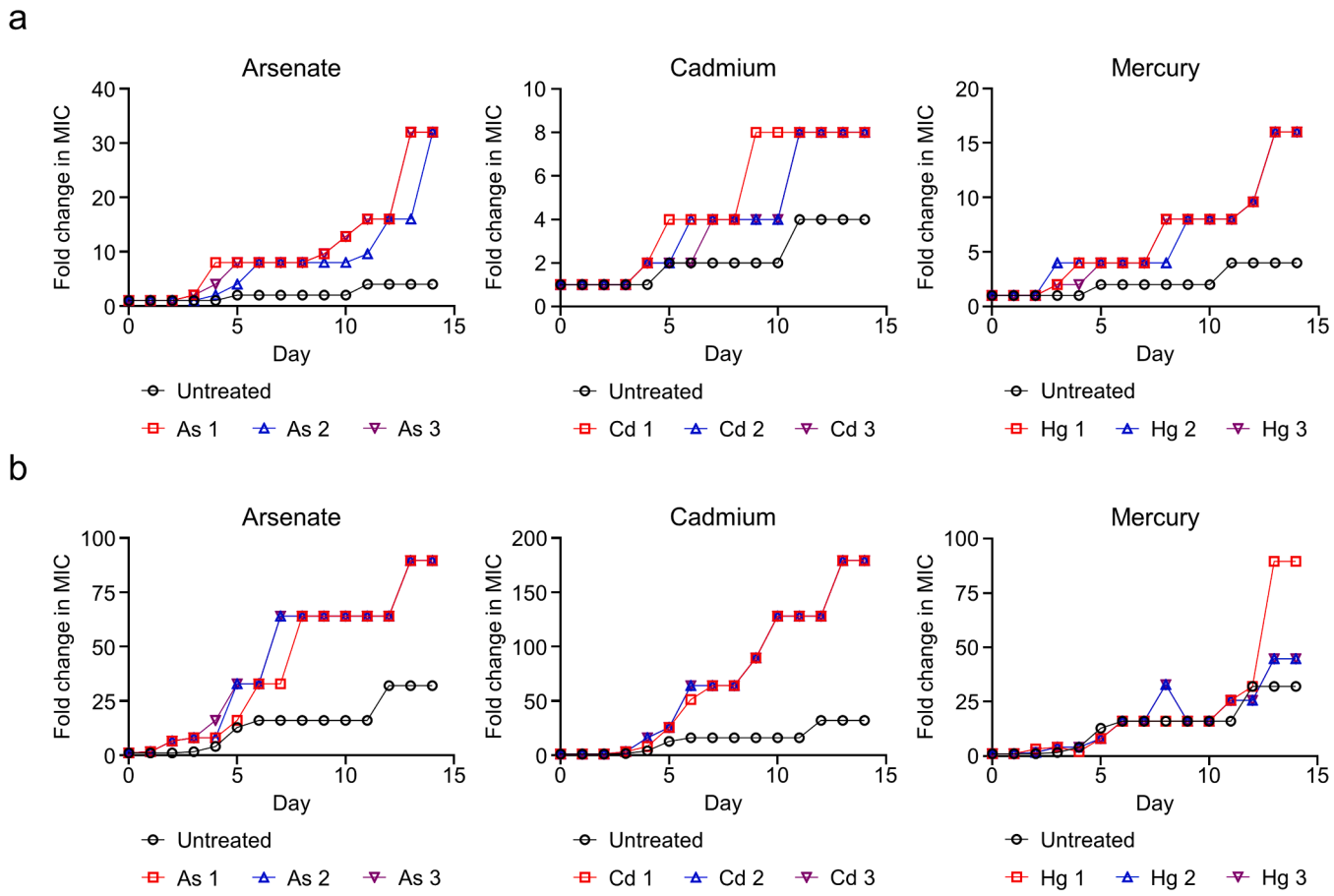


Fig. 7. Heavy metals increased the probability of resistance development. Emergence of ciprofloxacin resistance in *S. aureus* MW2 **(a)** and *E. coli* MG1655 **(b)** was monitored over 14 days of serial passage, with daily 30-min exposures to 4 × MIC of arsenate (As), cadmium (Cd), or mercury (Hg). Changes in ciprofloxacin MIC were measured at each passage to assess resistance development. Experiments were conducted in triplicate for each metal (As 1, As 2, As 3; Cd 1, Cd 2, Cd 3; Hg 1, Hg 2, Hg 3). Untreated refers to samples that were not exposed to heavy metals.

the heavy metal-induced persister cells of *S. aureus* MW2 and *E. coli* MG1655 to sub-MIC levels of ciprofloxacin. As resistance to ciprofloxacin can arise through *de novo* mutations, this antibiotic served as a useful tool for estimating resistance development probability (Friedman et al., 2006; Heisig and Tschorny, 1994). In untreated *S. aureus* MW2 cells, increase in the MIC for ciprofloxacin was up to 4-fold (Fig. 7a). Conversely, arsenate-, cadmium-, and mercury-induced *S. aureus* persisters exhibited increases in MIC of up to 32-fold, 8-fold, and 16-fold, respectively (Fig. 7a). Similarly, although untreated *E. coli* MG1655 cells showed an up to 32-fold increase in MIC during the 14-day passage, arsenate-, cadmium-, and mercury-induced *E. coli* persisters exhibited MIC increases of up to 92-fold, 188-fold, and 90-fold, respectively (Fig. 7b). These results suggest that heavy metal-induced persister cells have ROS-damaged DNA, which activates the SOS response, leading to DNA repair through error-prone polymerases. This process likely contributes to an elevated potential for resistance development.

4. Discussion

The "One Health" concept emphasizes the interconnectedness of the environment, animals, and humans in the spread of antibiotic resistance. As bacterial persisters serve as reservoirs for antibiotic resistance development, their formation should also be examined from a "One Health" perspective. Importantly, we demonstrate that exposure to heavy metals can cause substantial depletion of cellular ATP and generate ROS, transforming bacteria into a dormant state that is highly tolerant to antibiotics, known as persister cells. We showed that these persister cells are reprogrammed to survive in the presence of antibiotics, facilitating the development of antibiotic resistance. Our findings provide evidence that environmental pollutants, particularly heavy metals such as arsenic, cadmium, and mercury, promote the formation of bacterial persisters capable of tolerating high concentrations of three major classes of bactericidal antibiotics: DNA replication inhibitors, protein synthesis inhibitors, and cell wall synthesis inhibitors. The mechanisms underlying heavy metal-induced persister formation include growth arrest linked to ATP depletion and PMF dissipation, as well as the accumulation of intracellular ROS and activation of the SOS response. Notably, persister populations induced by heavy metal exposure exhibited an increased potential for developing antibiotic resistance.

We observed that arsenic and cadmium induced bacterial persister formation at relatively high concentrations of 5 mM (approximately 700 mg/kg) and 1.25 mM (approximately 140 mg/kg), respectively. Conversely, mercury promoted persister formation at a much lower concentration of 4 μM (approximately 0.8 mg/kg). Mercury levels sufficient to induce persister formation have been found in certain heavy metal-contaminated environments and marine organisms. For instance, mercury concentrations in sediment from Guanabara Bay, Brazil, which is polluted by domestic and industrial waste, have reached 37 mg/kg (Wasserman et al., 2000). Similarly, mercury levels in tilefish from the Gulf of Mexico have been determined to be 3.73 ppm, with an average of 1.45 ppm (Siddiqi et al., 2016). These concentrations are capable of inducing bacterial persister formation. Although the persister-inducing levels of arsenic and cadmium are generally too high to be found in

organisms, they have been detected at certain contaminated sites, including mines, and industrial sediments.

For example, the arsenic levels near a former gold mine in Złoty Stok, Poland, have exceeded 1000 mg/kg, with the maximum level reaching 4000 mg/kg (Stachnik et al., 2020). Moreover, soil samples from mining areas in Jinding, China, showed average cadmium concentrations of 175 mg/kg, with concentrations reaching 531 mg/kg (Wen et al., 2015). Additionally, a study conducted in the long-term heavy metal-contaminated Shibahe copper tailing dam area in Mountain Zhongtiaoshan, China, analyzed the correlation between soil microbial resistance and multi-metal contamination. The study reported that metal resistance genes (MRGs) and antibiotic resistance genes (ARGs) are potentially co-selected in heavy metal-contaminated soils (Chen et al., 2019). Furthermore, high levels of heavy metals and an increased abundance of ARGs in agricultural fields in villages near the Dabao Mountain mine in northern Guangdong province, southern China, led to substantial impacts on the microbial population (Zou et al., 2025). These findings suggest that the heavy metal concentrations in polluted sites or in contaminated organisms are likely sufficient to induce bacterial persister formation, potentially facilitating antibiotic resistance development. Bacteria are known to survive in heavy metal-contaminated environments through adaptive strategies, such as developing resistance, undergoing morphological changes, and producing exopolysaccharides (Mathivanan et al., 2021a). Our study demonstrates that persister formation could also be a critical survival strategy that bacteria employ under heavy metal stress.

ATP depletion is a well-known factor in persister formation in *S. aureus* and *E. coli*, as ATP is essential for the function of antibiotic targets and low ATP levels lead to metabolic dormancy (Niu et al., 2024). Consistently, we found that heavy metal treatment resulted in ATP depletion and PMF dissipation in both bacterial species. This is likely due to heavy metals disrupting the electron transport chain (ETC), impairing oxidative phosphorylation and reducing ATP synthesis. For example, mercury binds to sulfhydryl groups in ETC enzymes (Ajsuvakova et al., 2020; Fein et al., 2019), inhibiting their function and leading to energy loss. Additionally, heavy metals can compromise membrane integrity, causing ion leakage and further PMF dissipation (Warnes et al., 2012). As ATP depletion drives metabolic dormancy, this mechanism may explain how heavy metal exposure promotes persister formation and enhances antibiotic tolerance (Hossain et al., 2012; Xu and Imlay, 2012), which could explain the observed reductions in PMF and ATP levels in this study. Additionally, a recent study demonstrated that elevated ROS levels can disrupt iron–sulfur cluster-containing proteins, including those involved in the TCA cycle (Rowe et al., 2020). Deactivation of the TCA cycle impairs cellular respiration, subsequently reducing ATP production and inducing persister formation (Rowe et al., 2020). Our findings align with this, as we observed that heavy metal treatment increased the ROS levels in *S. aureus* and *E. coli*, suggesting that ROS accumulation may also contribute to ATP depletion and subsequent persister formation.

Accumulated ROS can cause damage to essential cellular components, including DNA and proteins, ultimately leading to cell death (Dwyer et al., 2015; Kohanski et al., 2010, 2007). However, several studies have shown that sublethal levels of ROS induced by antibiotics can promote antibiotic resistance development (Blázquez et al., 2018; Qi et al., 2023). Specifically, ROS such as hydroxyl radicals can cause DNA breaks and oxidation of base components, triggering SOS response for DNA repair. This repair process is mediated by a low-fidelity, error--prone DNA repair machinery, increasing the frequency and accumulation of mutations (Jaszczur et al., 2016; Qi et al., 2024, 2023). In our study, treatment with arsenic, cadmium, and mercury-induced intracellular ROS accumulation and upregulated the expression of *recA* and *umuC*, genes that encode the SOS response inducer and the error-prone polymerase V, respectively. This indicates that heavy metal-induced persister cells, which sustain DNA damage due to ROS, may activate the SOS response system. Consequently, when these persister cells are exposed to antibiotics, the activated low-fidelity, error-prone DNA repair machinery may promote mutations that confer antibiotic resistance. This mechanism could explain why heavy metal-induced persister cells have a higher likelihood of developing resistance compared to normal cells.

Our study is in line with previous reports showing the interconnection between responses to heavy metals and antibiotics. Bacteria possess efflux pumps, metal sequestering proteins, and detoxification systems to mitigate heavy metal toxicity (Mathivanan et al., 2021b). Some of these mechanisms overlap with those involved in antibiotic resistance, suggesting a potential link between heavy metal adaptation and antibiotic tolerance. For example, efflux pumps such as AcrAB-TolC and NorA, which export antibiotics, have also been implicated in metal resistance (Sun et al., 2014). The co-regulation of these pathways may enhance bacterial survival under metal stress while inadvertently promoting antibiotic resistance. Additionally, the induction of stress response regulators, such as SoxS, in response to heavy metal exposure could further modulate persister formation and resistance evolution (Chukwu et al., 2023). Understanding these overlapping response strategies may provide novel insights into how environmental pollutants drive the persistence and adaptation of bacterial populations.

5. Conclusions

Our findings demonstrate that heavy metal toxicity can generally induce bacterial persisters through mechanisms involving ATP depletion and ROS accumulation. This underscores the substantial selective pressure that heavy metals impose on bacterial populations, promoting antibiotic resistance through the SOS response. Moreover, the study results highlight the importance of considering heavy metals in the broader context of bacterial persistence and resistance. Understanding these interactions is critical for developing strategies to address the complex challenges associated with bacterial persistence and spread of antibiotic resistance. Our findings further emphasize the need for a comprehensive “One Health” approach to tackle the growing threat of antimicrobial resistance.

CRediT authorship contribution statement

Chang JuOae: Writing – review & editing, Methodology, Investigation. **Shin YuJin:** Methodology, Investigation. **Choi Jiwoo:** Methodology, Investigation. **Yun Taegwan:** Methodology, Investigation. **Kim Jin:** Methodology, Investigation. **Baek Seongeun:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Seo Jinbeom:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Kim Wooseong:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Lee Wonsik:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Kim Inseo:** Methodology, Investigation. **Yang Yung-Hun:** Writing – review & editing, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118014.

Data availability

Data will be made available on request.

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