Journal of Hazardous Materials 369 (2019) 9–16

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Sub-lethal concentrations of heavy metals induce antibiotic resistance via mutagenesis

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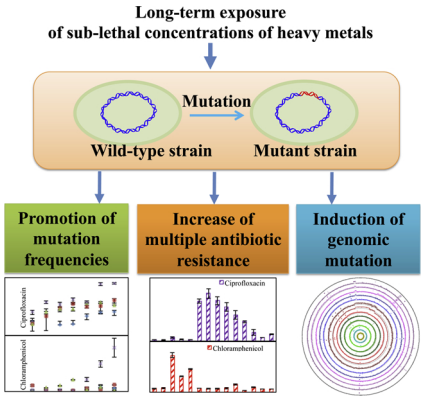
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Sub-lethal concentrations
Heavy metals
Antibiotic resistance
Mutation

ABSTRACT

The emergence of antibiotic resistance is a growing problem worldwide. Numerous studies have demonstrated that heavy metals facilitate the spread of bacterial drug-resistance in the environment. However, the actions and mechanisms of metals at relatively low sub-lethal levels (far below the minimal inhibitory concentration [MIC]) on antibiotic resistance remain unclear. In this study, we investigated the effect of sub-lethal levels of heavy metals [Ag (I), Zn(II), and Cu(II)] on antibiotic resistance and explored the underlying mechanisms. The results demonstrated that sub-lethal levels of metal ions increased the mutation rates and enriched *de novo* mutants that exhibited significant resistance to multiple antibiotics. The resistant mutants exhibited hereditary resistance after 5-day of subculture. Whole-genome analysis revealed distinct mutations in genes involved in multiple drug and drug-specific resistance, as well as genes that are not associated with antibiotic resistance to data. The number and identities of genetic changes were distinct for mutants induced by different metals. This study provides evidence and mechanistic insights into the induction of antibiotic resistance by sub-lethal concentrations of heavy metals, which may enhance the emergence of antibiotic resistance in various environments. More consideration and regulations should be given to this potential health risk for long-standing and harmful heavy metals.

Abbreviations: ARGs, antibiotic resistance genes; ARB, antibiotic resistant bacteria; MIC, minimal inhibitory concentration; Sub-MIC, sub-lethal concentration

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https://doi.org/10.1016/j.jhazmat.2019.02.006
Received 23 September 2018; Received in revised form 31 January 2019; Accepted 3 February 2019
Available online 04 February 2019
0304-3894/

1. Introduction

Antibiotic resistance has become a serious threat to global public health [1]. In addition to the overuse and misuse of antibiotics, heavy metals can accelerate the emergence and spread of antibiotic resistance [2–6]. Numerous observational studies have shown that heavy metals enrich antibiotic resistance in various environments, where the abundance of antibiotic resistance genes (ARGs) were significantly correlated with the concentration of heavy metals [2,3,7,8]. For example, sulfonamide resistance genes (*sulA* and *sulIII*) were strongly correlated with the levels of Cu, Zn and Hg in the soil [2]. In addition, tetracycline resistance genes were shown to be associated with the abundance of As and Cu in manure samples collected from swine farms [3], as well as high Cu exposure co-selected for increased community-level resistance to tetracycline and vancomycin in the soil [7]. The co-selection of bacterial resistance to metals and antibiotics is driven by two mechanisms, including co-resistance (genes conferring resistance to metals and antibiotics are simultaneously distributed in a mobile genetic element, such as a plasmid) and cross-resistance (antibiotic and metal use the same resistant pathway, e.g., multidrug resistance efflux pumps) [5,6].

Chromosomal mutation is the fundamental mechanism for bacteria to obtain ARGs, which refers to the induction of antibiotic resistance via base substitution or frameshift in specific genes [9–12]. Several studies have revealed that sub-lethal concentrations (far below the minimal inhibitory concentrations [MICs], also referred to as sub-MICs or sub-inhibitory concentrations) of antibiotics result in increased mutational space in different chromosomal loci for bacteria to form a wide range of mutant phenotypes. Moreover, these phenotypes occur at a high frequency because of the low fitness-cost of mutations compared with those that result from exposure to a lethal dosage (greater than MICs) of antibiotics [11,13]. This phenomenon suggests that sub-lethal levels of antibiotics are beneficial for the acquisition of ARGs [9,11,13].

In previous studies, radical-induced mutagenesis has been shown to be one of the dominant mechanisms through which bacteria develop antibiotic resistance; such mutagenesis is triggered by sub-lethal levels of antibiotics and disinfectants [9,11]. Heavy metals, which are also used as antimicrobial agents, exhibit mechanisms of action similar to those of antibiotics on certain pathways [14,15]. For example, heavy metals (e.g., Ag, Cu, Cr, and Cd) can kill bacteria or inhibit bacterial growth by inducing oxidative stress and DNA damage in vivo, which can also induce mutagenesis and genotoxicity [14,15]. However, owing to the current focus on high-level (> MIC) selection for metal resistance [6], the effect of sub-lethal concentrations of metals on the induction of antibiotic resistance has been largely ignored.

In contrast to the short half-lives and biodegradable nature of antibiotics, heavy metals are non-biodegradable and remain in environments for extended periods of time. Metals are extensively persistent at relatively low concentrations (below their MICs) in various environments [16–19]; therefore, metals may exert long-term selective pressures for antibiotic resistance. Additionally, many metals, such as Cu and Zn, are widely used in feeding additives; therefore, bacteria are often in close contact with low concentrations of various metals in the gut and in manure [3,14]. Hence, whether the long-standing and sub-lethal concentrations of heavy metals can promote antibiotic resistance represents a significant concern for public health.

Recently, several reports demonstrated that a very low level of metals (far lower than the MIC) enabled bacteria via co-selection to obtain a resistant plasmid, which harbors heavy metal and antibiotic resistance genes [4,20]. However, to our knowledge, few studies have focused on the enrichment of antibiotic resistant bacteria (ARB) through chromosomal mutations induced by sub-MIC levels of metals. Therefore, in this study, we simulated the effect of sub-lethal levels of heavy metals through evolutionary experiments [21] to investigate whether sub-lethal levels of heavy metals can significantly enhance the generation of antibiotic resistance through genetic mutations. In addition, whole-genome sequencing was performed to explore the molecular mechanisms underlying the antibiotic resistance of these mutants. Our results could potentially reshape the overall understanding of the evolution and spread of antibiotic resistance and improve the health risk assessment and management of heavy metals in the environment and feed additives.

2. Materials and methods

2.1. Bacteria strains, culture conditions, antibiotics, and metals

The *Escherichia coli* strain MG1655 K12 (denoted as wild-type strain *E. coli*) was revived from a −80 °C glycerol stock by streaking the culture onto Luria-Bertani (LB) agar plates, and a single colony of *E. coli* was selected and used as the seed strain for the subsequent experiments according to previous studies [22]. All antibiotics (amoxicillin, ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamycin, tetracycline, and trimethoprim) and heavy metals ($AgNO_3$, $CuSO_4{\cdot}5H_2O$, and $ZnSO_4{\cdot}7H_2O$) were purchased from Aladdin Reagent Database, Inc. (Shanghai, China).

2.2. Minimum inhibitory concentrations (MICs) determination

The MICs of each antibiotic and metal ion against the original *E. coli* K12 and its derivative strains isolated from the following exposure experiments were determined according to previous studies [22,23], and detailed descriptions are presented in Text S1.

2.3. Exposure experiments with sub-lethal concentrations of heavy metals

The exposure experiments were initiated with the isogenic *E. coli* K12 cultures, and the detailed procedures are illustrated in Fig. S1 as previously described [21,22]. Initially, 1 mL of isogenic *E. coli* cultures (approximately 10^8 CFU) and 4 mL of fresh LB medium with the indicated concentrations of a given metal ions (Table S1) were added into a 15 mL sterilized tube. The cultures were incubated at 37 °C for 24 h, and then 1 mL of *E. coli* cultures from this tube was sub-cultured in 4 mL fresh LB medium with the corresponding metals. Isogenic *E. coli* incubated in LB medium without any metal ions was also cultured in parallel, serving as the control group. All experimental treatments and the control treatment were performed in triplicate. We referred to the initial culture of bacteria with metal treatments or without any metal treatment (control group) as cycle 0 (C0), and serial sub-cultures were designated C1, C2, etc. (Fig. S1). During the serial sub-cultures, 3 mL of the culture of each evolutionary cycle was stored at −80 °C in a 15% (v/v) glycerol solution for further analysis. All the experimental tubes were incubated in aerated incubators with 200 rpm shaking, and the selection experiments were repeated every 24 h for 54 sub-culture cycles (Fig. S1).

2.4. Determination of the mutation rates, isolation of resistant mutants and determination of minimal inhibitory concentrations (MICs)

The mutation rates were determined every 9 cycles of sub-culture during the treatment experiments as described previously [9,12]. To identify the resistant mutants in the metal-treated cultures and control groups, sub-culture aliquots were used to coat on LB agar plates with 4 different antibiotics (ampicillin, chloramphenicol, ciprofloxacin and tetracycline) at 1 × MIC (Table S2), and then, the plates were incubated for 48 h at 37 °C [9,13]. The total bacterial concentrations were determined by plating on LB agar. The multiple solution scheme (MSS) maximum-likelihood method was employed to estimate the concentrations of the mutations in each culture [24]. Mutation rates were determined by dividing the number of resistant clones by the total number of bacterial cells. It was assumed that the resistant clones generated after normalization to the control group were attributed to

mutagenesis induced by heavy metals [9,12]. Fold changes in the mutation rate were determined for each chemical treatment relative to an untreated control group.

To determine the resistance phenotype of the mutants to different antibiotics, three resistant mutational clones were randomly selected from each treatment after 54 selection cycles and one clone was isolated from the control groups. Given the lack of a significant increase in the mutation frequency of *E. coli* resistant to ampicillin and tetracycline following treatment with the three metals compared with controls, we did not select mutant clones from the LB plates containing these antibiotics. The above resistant colonies were regrown in 5 mL of fresh LB medium at 37 °C for 6 h for MIC determination as previously described (TextS1).

2.5. Hereditary stability of the resistant mutants

The selected resistant clones were cultured in 5 mL of LB broth without any antibiotics or heavy metals at 37 °C with shaking at 180 rpm for 24 h. Then, the cultures were diluted 1:100 in 5 mL of fresh LB and incubated again. Each mutant was subjected to five rounds of sub-culture as described in previous studies [9,12]. The MICs of the initial mutants and the cultures at the end of five rounds of sub-culture were measured to determine whether the resistance was maintained.

2.6. DNA Extraction, whole-genome sequencing and data analysis

The genetic changes in the resistant mutants were analyzed by whole-genome sequencing according to our previous study [22]. Detailed descriptions of the DNA extraction, whole-genome sequencing and data analysis are presented in Text S2.

2.7. Statistical analysis

Analysis of significant differences was performed by using analysis of variance (ANOVA) and an independent sample *t*-test (SPSS 18.0, Chicago, USA). A *p*-value of < 0.05, 0.01, and 0.001 was considered as significant, very significant, and extremely significant, respectively [25].

3. Results and discussion

3.1. Sub-lethal concentrations of heavy metal ions increase mutation rates

The MICs of Ag(I), Zn(II), and Cu(II) against the initial drug-sensitive wild-type *E. coli* K12 were 8.95, 134 and 575 mg/L, respectively (Table S1). Based on the MICs detected in the present study, the concentrations listed in the Chinese Environmental Quality Standard for Soils (GB15618-1995) and the Wastewater Discharge Standard (GB8978-1996), and the environmental concentrations of these metal ions [16,26], a series of sub-lethal concentrations (Table S1) was selected and applied in the following exposure experiments. It should be noted that the MICs of the metals against *E. coli* K12 were measured using LB medium, in which parts of metal ions may be absorbed by its components. However, because all experiment groups and the control group were constructed using LB medium, the impact of the absorbed dosages on experiments could be balanced out.

The spontaneous mutation rates of the control group without exposure to any metals ranged from 10^{-9} to10^{-7} mutations/cell/generation for various antibiotics (data not shown), which was consistent with previous studies [9,13]. The mutation rate of *E. coli* that was resistant to ciprofloxacin (fluoroquinolone antibiotic) gradually increased over time following exposure to sub-lethal levels of Cu(II), Zn(II) and Ag(I), and the increase was 7–20, 4–27 and 14.6–1538.2 folds, respectively, compared with the control group (Fig. 1A). However, only treatment with sub-lethal levels of Ag(I) resulted in a significant increase in the mutation frequency for chloramphenicol resistance

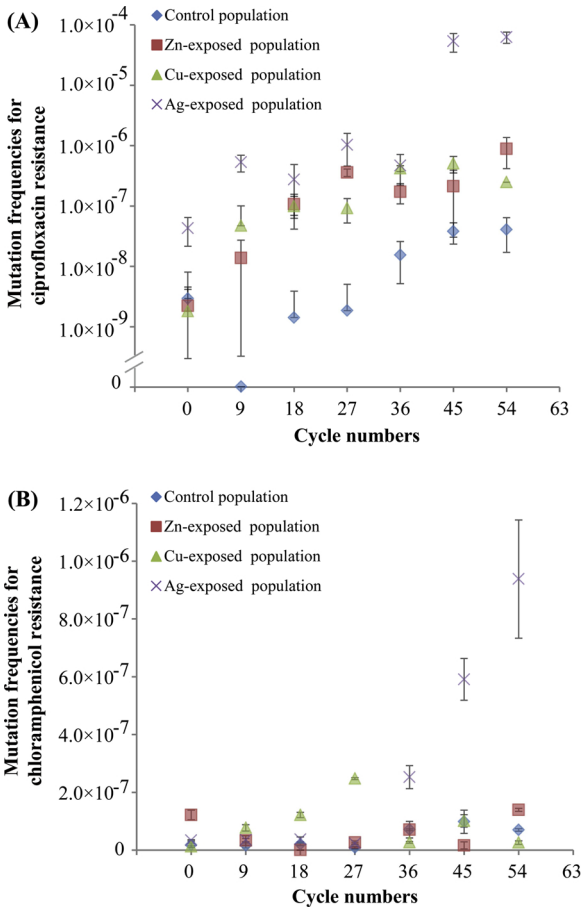

Fig. 1. Selection of *de novo* resistant mutants at sub-lethal concentration of metals. A total of 12 independent lineages of *E. coli* were serially passaged in LB medium without metal amendment (controls) or supplemented with a final concentration of Zn (26.81 mg/L), Cu (11.50 mg/L), or Ag (1.79 mg/L). Every 9 cycles, cultures were screened for resistant bacteria using LB agar plates with ciprofloxacin (A) and chloramphenicol (B), and the ratio of resistant bacteria was calculated. The data points are grouped by the number of cycles of growth and the resistance level; in each of these datasets, one data point represents the average fraction of cells present in one lineage that is capable of growth under 1 × MIC of each antibiotic, and error bars represent the standard deviation of at least three replicates.

(Fig. 1B). In addition, these three metal ions did not induce significantly increased mutation rates of *E. coli* that were resistant to other antibiotics (ampicillin and tetracycline) compared with the controls (data not shown).

Comparing the mutation patterns for ciprofloxacin and chloramphenicol, we found that the mutation rate for ciprofloxacin resistance induced by all three metals increased over time, whereas the mutation rate for chloramphenicol resistance induced by Ag(I) increased significantly after 36 sub-cultures (Fig. 1). This finding suggests that resistance to ciprofloxacin occurs through adaptive mutations confined to a smaller genomic region, whereas resistance to chloramphenicol may require mutations in a diverse set of genes [27].

The present results indicated that these sub-lethal levels of metal ions (likely higher than the actual concentrations due to parts of metals absorbed by LB medium's components) could rapidly enrich *de novo* resistant mutants. Previous evidence also suggested that very low concentrations of antibiotics [9,13], disinfectants [9], and disinfection by-products (DBPs) [12,22] could select for and induce resistant mutants. Thus, along with previous studies [4,20], we speculated that the emergence of antibiotic-resistant bacteria in various environments is likely to be partly explained by the selective effects of sub-lethal concentrations of environmental chemicals.

3.2. Sub-lethal concentrations of heavy metal ions increase multiple antibiotic resistance

Compared with the original *E. coli* K12 strain before exposure to heavy metals, the random isolate from the control group grown in LB medium did not exhibit significant changes in the MICs (Fig. 2). However, the sub-lethal levels of the three metals [Ag(I), Cu(II), and Zn (II)] induced mutational clones (designated as Ag-CIP-1–3, Cu-CIP-1–3, and Zn-CIP-1–3) that exhibited increased resistance to ciprofloxacin with an approximate 24.8-, 15.6- and 3.9- fold increase in the MICs, respectively, compared with the control group (Fig. 2A). The Ag(I)-induced mutants (designated as Ag-CHL-1–3) exhibited significant resistance to chloramphenicol with a 5.5- to 11.7- fold increase in the MICs compared with the control group (Fig. 2B). Given that non-biodegradable heavy metals are extensively persistent at relatively low concentrations (below their MICs) in various environments [16–19], the present results implied that environmental heavy metals may exert long-standing pressure for the enrichment of antibiotic resistance populations.

These resistant mutants also exhibited multiple resistances (Figs. 2 and S2), which is more threatening to public health than mutants that are resistant to a single antibiotic [28,29]. For example, chloramphenicol resistant mutants induced by sub-lethal levels of Ag(I) also exhibited significant resistance to ampicillin, tetracycline and ciprofloxacin, and the Zn(II)-evolved ciprofloxacin resistant mutants also exhibited resistance to chloramphenicol and tetracycline (Figs. 2B and S2). These results were consistent with previous reports indicating that strains that evolved in the presence of chloramphenicol exhibited increased resistance to doxycycline (belonging to class of tetracycline resistance) [27] and tetracycline [30]. Resistance to multiple antibiotics can develop when the same resistance mechanisms is used for multiple antibiotics, such as changes in cell membrane permeability, the activation of efflux pumps, the modification of antibiotic target sites, and structural changes or degradation of the antibiotics [27,31].

3.3. Hereditary antibiotic resistance induced by sub-lethal levels of heavy metals

Hereditary antibiotic resistance represents a significant issue for public health [9,12]. As shown in Fig. 2, the MICs of 9 mutants, including six out of nine ciprofloxacin resistant mutants and all three chloramphenicol resistant mutants, were significantly altered after a 5-cycle sub-culture. Specifically, the resistance level to ciprofloxacin/chloramphenicol in 8 mutants significantly decreased, and conversely, the resistance level in 4 mutants increased significantly. This phenomenon may be partly attributed to genotypic-phenotypic discrepancies [32,33]. For bacteria exposed to antibiotics, an increased or decreased resistance level can be achieved by the combination of phenotypic changes (phenotypic resistance, not-inheritable resistance) and genetic changes (inheritable resistance) [32]. Therefore, we speculated that the significant changes in the resistance level to antibiotics in 9 mutants were likely the consequences of the loss of phenotypic changes after 5 rounds of sub-culture in metal-free medium. Nevertheless, all evolved strains maintained a significantly increased ciprofloxacin/chloramphenicol resistance compared with the initial *E. coli* K12 and the control strain; this increased resistance was achieved through genetic changes, according to the genome sequencing analysis in Section 3.4. In summary, these results suggested that the mutant strains could stably pass on the resistance to their offspring. In addition, the fitness costs of the mutations were probably low under a weak selective pressure [11]. Given that bacteria can proliferate and spread in various environments, hereditary resistance may result in the spread of antibiotic resistance [34].

3.4. Mechanisms underlying the induction of antibiotic resistance by sub-lethal levels of heavy metals

To identify the genetic changes responsible for the antibiotic resistance induced by sub-lethal levels of heavy metals, we performed whole-genome sequencing of the resistant mutants. As described above, resistant mutants from the final cycle of the evolutionary experiments isolated by spreading the cultures on LB plates containing ciprofloxacin (Ag-CIP-1–3, Cu-CIP-1–3, and Zn-CIP-1–3) and chloramphenicol (Ag-CHL-1–3) and one clone from the control group (denoted as control) were randomly selected and subjected to whole-genome sequencing. These sequencing reads were deposited in the NCBI Sequence Read Archive under accession number SRP082640. Due to the poor quality of the sequencing data for Cu-CIP-2, this strain was excluded from further analysis. In total, 96 genetic changes, including 86 SNPs and 10 InDels (Fig. 3, Table S3), were identified compared with the initial wild-type *E. coli* K12 strain (GeneBank Accession Number: NC_000913.3). These genetic changes were located within 17 genes (16 protein coding genes and 1 RNA encoding gene) and 11 putatively intergenic regions in the genome (referred to as IGR) (Fig. 3, Table S4). These genes were categorized into the following four major functional groups: (1) transcription and translation (*gyrA*, *rpoB*, *rpoA*, *lysV*, *rsmD*, *cytR*, *rbsR* and *atoC*); (2) cell wall structure (*mreB*, *mpl*, *fimA* and *dacA*); (3) membrane transport (*trkH* and *icd*); and (4) unknown function (*rzpD*) (Fig. 4, Table S4).

All these three metals induced genetic changes in the *gyrA* genes, which is involved in transcription and translation (Figs. 3 and 4). Two distinct amino acid substitution mutations were identified in the *gyrA* gene, including a G→A substitution in base 2,339,173, causing a Ser-83→Leu missense mutation in the resistant strains selected by Ag(I) and Cu(II) (Ag-CIP-1, Cu-CIP-1 and Cu-CIP-3 strains), and a C→T

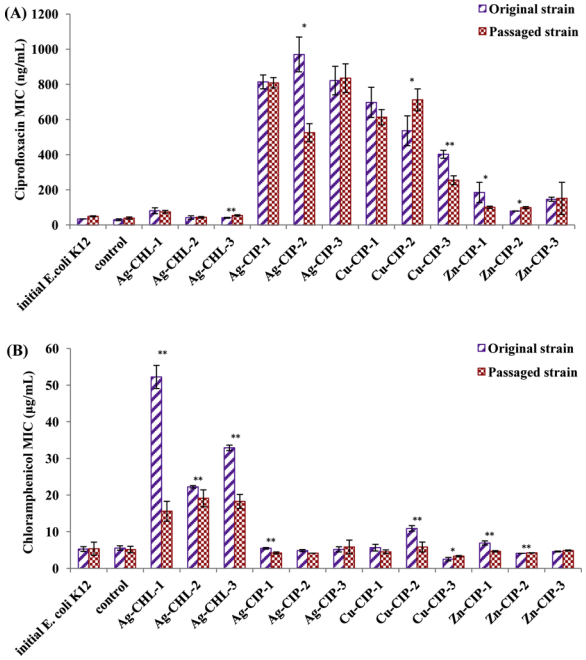

Fig. 2. Determination of MICs of ciprofloxacin (A) and chloramphenicol (B) for 12 evolved mutants derived from populations under metal exposure. The passaged strain is defined as the original strain (mutant, initial *E. coli* K12 or control strain) that was serially passaged for 5 days every 24 h in LB medium without any antibiotics and heavy metals. Ag-CHL-1–3, chloramphenicol mutants with sub-MIC of Ag(I) selection; Ag-CIP-1–3, Cu-CIP-1–3 and Zn-CIP-1–3, the evolved ciprofloxacin mutants by exposure to sub-MIC of Ag(I), Cu(II) and Zn(II), respectively; control, a single clone randomly isolated from the metal-untreated culture. Significant differences were tested with independent sample *t*-test and shown with * ($P < 0.05$) and ** ($P < 0.01$).

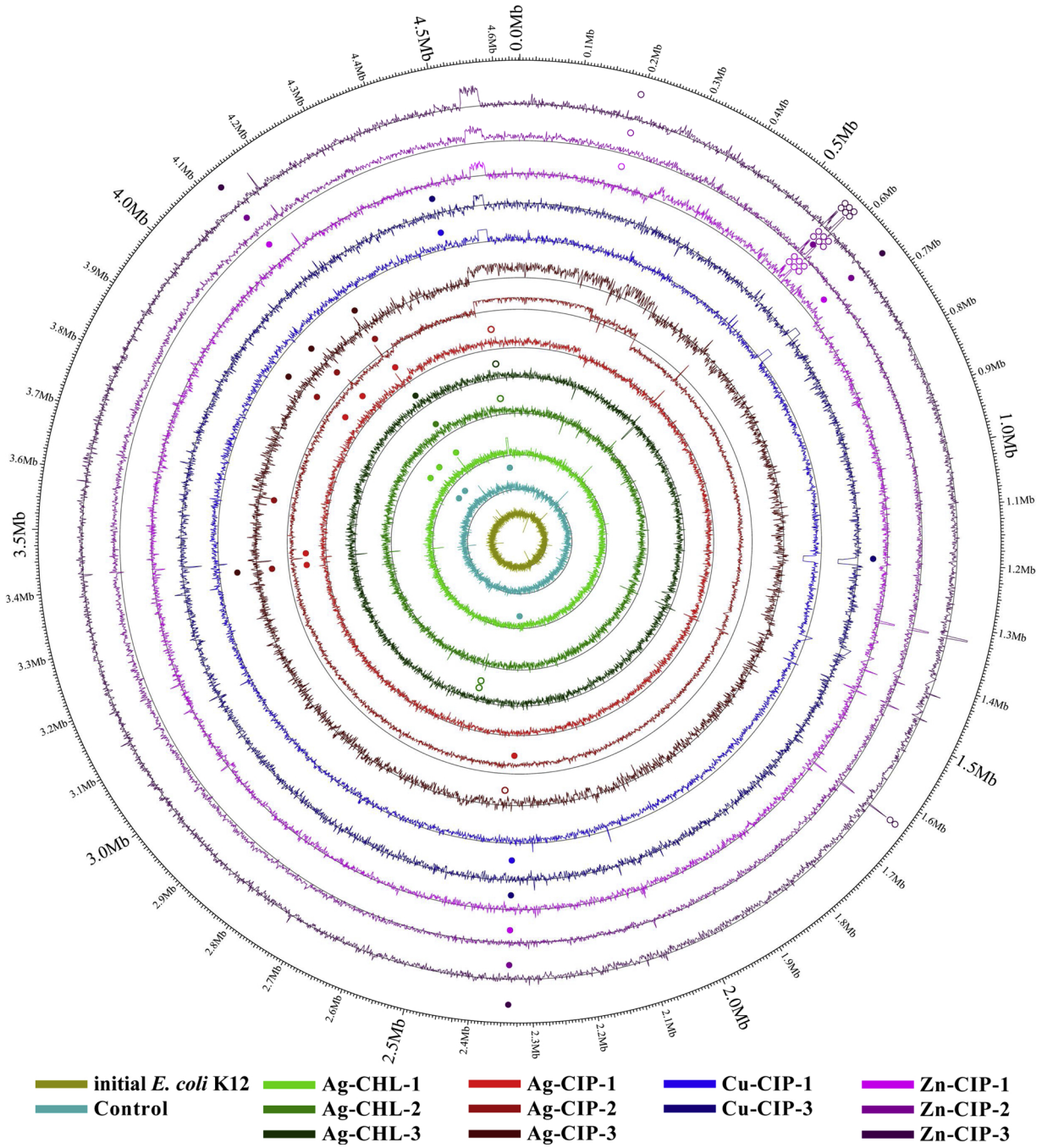

Fig. 3. Comparison of genome coverage and mutation distribution among the evolved antibiotic-resistant mutants, the initial *E. coli* K12 and the control. Sequencing coverage of each strain was plotted using a different line color, and the corresponding dark gray line in each ring represents the average coverage. Solid dots and hollow dots indicated that the SNP/InDel occurred in the gene and intergenetic region (IGR), respectively. In addition, synonymous mutations occurred in gene were not shown in this Figure. Detailed information on the mutations is shown in Table S3 and Table S4.

substitution in base 2,339,162, causing an Asp-87→Asn missense mutation in the resistant strains selected by Zn(II) (Zn-CIP-1–3 strains) (Figs. 3 and 4, Table S4). The *gyrA* gene encodes DNA gyrase, which is the target of flurorquinolone antibiotics (e.g., ciprofloxacin and nalidixic acid) [35]. Previous evidence indicated that ciprofloxacin inhibits *gyrA* function by blocking in the active site of the enzyme [35–37]. Correspondingly, amino acid variations in the *gyrA* subunit of bacteria are associated with resistance to ciprofloxacin [36,37]. Several studies have indicated that mutations in *gyrA*, especially at Ser-83, are frequently found in ciprofloxacin-resistant *E. coli* strains [35–37]. The two genetic changes in *gyrA* gene identified in this study are also likely to contribute to ciprofloxacin resistance.

All of the Ag(I)-induced resistant strains (Ag-CIP-1–3 and Ag-CHL-1–3) carried an SNP (C→A substitution in base 4,183,652, causing an Ala-803→Glu missense mutation) in the *rpoB* gene, which encodes the RNA polymerase β subunit; *rpoB* mutations are involved in chloramphenicol resistance [30]. In addition, sub-lethal levels of Ag(I) also stimulated mutations in *rpoA* (a C→T SNP in base 3,441,023 causing a Gly-3→Ser missense mutation), *lysV* (a T→C SNP in base 2,521,268 and a T→G SNP in base 2,521,272), and *rsmD* (an GCCC→GCCCAGCC insertion in base 3,604,481, causing a frameshift mutation) (Fig. 4, Table S4). The *rpoA* gene encodes a protein homologous to the alpha subunit of RNA polymerase, and it is known to play a role in rifampicin resistance in *E. coli* [38] and *Mycobacterium tuberculosis* [39]. A deletion

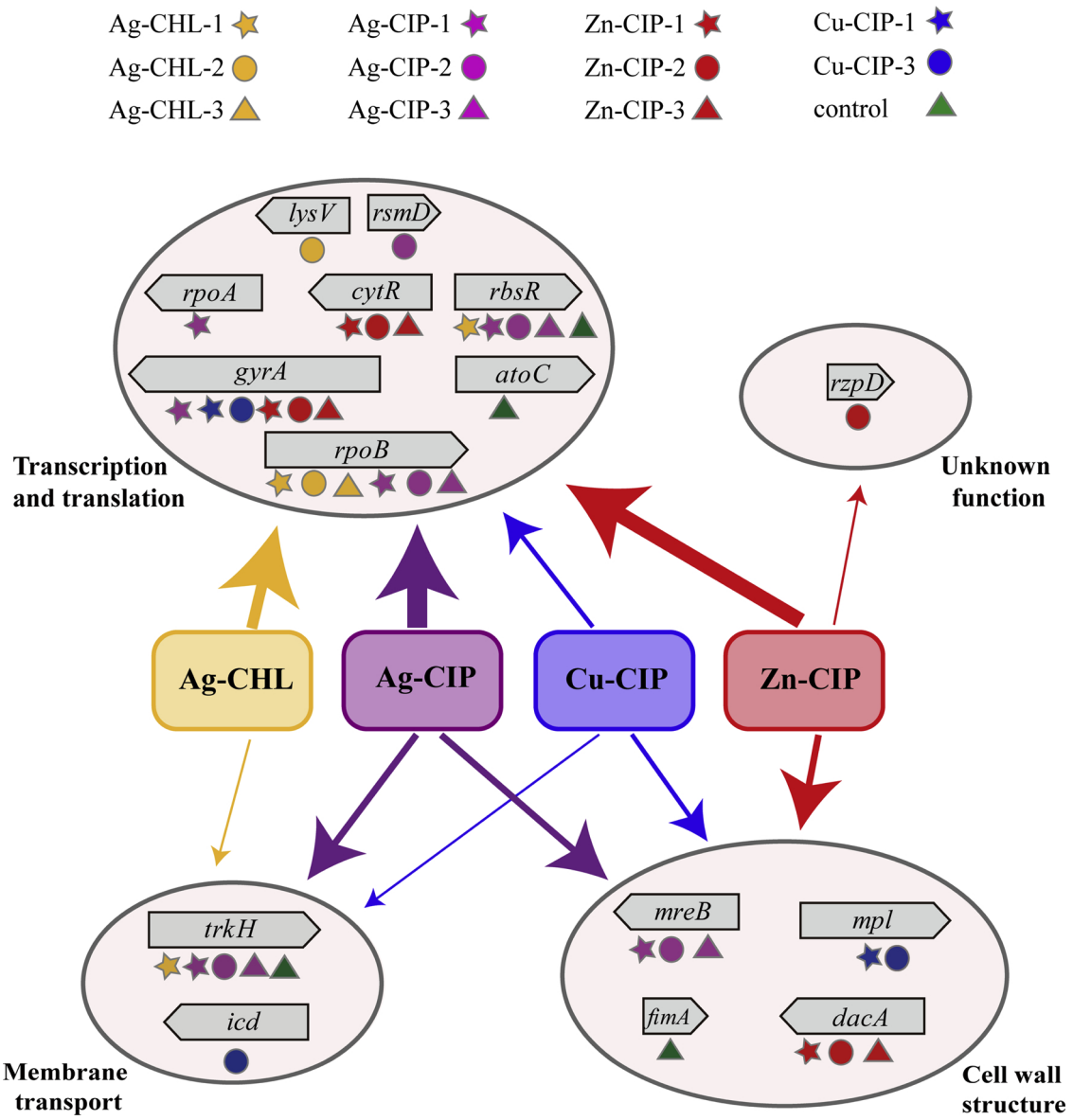

Fig. 4. Unique mutations identified by whole-genome sequencing. The evolved strains are denoted by different symbols, with colors indicating different categories of the evolved strains. The horizontal arrow blocks denote the genes. Mutations identified in each of the evolved strains are shown with the corresponding symbols appended to the genes. These genes clustered into the following four major functional groups: (1) transcription and translation, (2) cell wall structure, (3) membrane transport, and (4) unknown function. The arrow thickness reflects the frequency of the mutations occurring within each functional group for each category of the evolved strains.

mutation in the *lysV* gene was previously discovered in antibiotic resistant *E. coli*; however, it is unknown whether this gene is related to antibiotic resistance [30]. RsmD exhibits m2G966 methyltransferase activity for 16 S rRNA in *E. coli* [40], but this protein has not been reported to be involved in ciprofloxacin and chloramphenicol resistance. Ag-CIP-1–3 and Ag-CHL-1 exhibited common amino acid substitutions, i.e., Arg-307→Gln in *rbsR*, indicating its close association with antibiotic resistance [41]; however, a mutant also occurred at a different site (Gln-255→* in *rbsR*, leading to a truncated mutation) in the control group.

It is notable that all three metals induced genetic changes that were associated with cell wall related genes, namely, *mreB*, *mpl* and *dacA* genes (Fig. 4, Table S4). The MreB protein is a crucial component of the cell wall structural complex MreBCD, which is responsible for determining of the cell shape and the sensitivity of cell growth to amidino penicillin, mecillinam and fluoroquinolones [35,42]. Therefore, the mutation in the *mreB* gene (a G→C SNP in base 3,400,627 causing a Pro-154→Arg missense mutation) induced by Ag(I) may be responsible for the relatively high level of resistance to ciprofloxacin (Fig. 4).The *mpl* gene encodes UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-2, 6-diaminoheptanedioate ligase, which is an enzyme that is essential for recycling of cell wall peptidoglycan [40,43]. This enzyme is typically the cellular target of various antibiotics, such as cefoperazone [40]. Cu(II)-evolved mutants carried a deletion mutation in *mpl* (7 bases 'GTGGCAG' deletion in base of 4,456,970, causing a frameshift mutation at 396th aa), which may contribute to antibiotic resistance. The *dacA* gene, which encodes D-alanyl-D-alanine carboxypeptidase, plays an important role in the intrinsic beta-lactam resistance of *E. coli* [44].

Sub-lethal levels of Ag(I) and Cu(II) also induced the same genetic changes associated with membrane transport genes (*trkH* and *icd*) (Fig. 4, Table S4). The TrkH protein mediates K + uptake in bacteria and is likely evolved from simple K + channels by multiple gene duplications or fusions [45]. Previous evidence demonstrated that the

K + uptake protein is required for serum, protamine, and polymyxin B resistance in *Vibrio vulnificus* [46]. We detected two distinct genetic changes in the *trkH* gene occurred in strains exposed to Ag(I) (a G→A SNP in base of 4,033,217 causing a Gly-25→Arg missense mutation) and the control group (a A→G SNP in base of 4,033,616 causing a Met-158→Val missense mutation). In addition, two SNPs (T→G in base 2,322,251 in *atoC* and G→A in base 4,543,367 in *fimA*) were also identified in the control group (Figs. 3 and 4, and Table S4). The lack of significant changes in the MIC of ciprofloxacin in the control group (Fig. 2) indicated the possibility of spontaneous mutations (resulting from incubation conditions and potential stress) [22]. Additionally, Cu-CIP-3 harbored an Asp-398→Glu substitution in *icd*. This gene encodes isocitrate dehydrogenase, which functions in the tricarboxylic acid cycle, and the abolition of ICDH activity significantly affects the respiratory system and the electron transport chain [47]. In addition, Zn-CIP-3 contained a Val-35→Ala substitution in *rzpD*. This gene may play a role in antibiotic resistance, as it is involved in biofilm formation in *E. coli* [48].

In the present study, the mutations resulting from the evolutionary adaptation to sub-MIC levels of metals were not directly affected by antibiotics. Thus, not all mutated genes identified in the evolved strains may be responsible for their antibiotics resistance. Few mutations identified in this study overlap with previous studies, which may be due to the random mutations generated in response to exposure to metal pressures [27,30,49]. Furthermore, the increase in the MIC of ciprofloxacin or chloramphenicol was less than that previously reported in the evolved lineages upon exposure to antibiotics (a 32- to 192-fold increase in the MIC of ciprofloxacin) [50], possibly suggesting a weak selective pressure of sub-MIC levels of metals on bacterial antibiotic resistance compared with antibiotics. However, the inheritable and long-standing mutations also pose important roles in the emergence and spread of antibiotic resistance in the environment.

4. Conclusion

To date, understanding how the evolution of bacteria exposed to sub-MIC concentrations of a given metal influences antibiotic resistance represents an important public health concern. In this study, by combining evolutionary experiments and a whole-genome sequencing approach [51], we revealed that long-term exposure to sub-MIC levels of certain metals increases the frequency of bacterial resistance to antibiotics via genetic mutations. This finding suggests that the low levels of metal in many natural environments impose a widespread selective pressure on bacteria, which most likely accelerates the emergence and spread of resistant bacteria [11]. Thus, more attention should be given to the potential health risk of poisonous and harmful heavy metals.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 21507012, 21477024, 91643106, and 21527814), China Postdoctoral Science Foundation (No. 2015M570329), and the Natural Science Foundation of Guizhou Provincial Department of Education of China (No. KY[2015]395).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.02.006.

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