



Occurrence and dissemination of antibiotic resistance genes in mine soil ecosystems

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Abstract

Metal(loid) selection contributes to selection pressure on antibiotic resistance, but to our knowledge, evidence of the dissemination of antibiotic resistance genes (ARGs) induced by metal(loid)s in mine soil ecosystems is rare. In the current study, using a high-throughput sequencing (HTS)-based metagenomic approach, 819 ARG subtypes were identified in a mine soil ecosystem, indicating that these environmental habitats are important reservoirs of ARGs. The results showed that metal(loid)-induced coselection has an important role in the distribution of soil ARGs. Furthermore, metal(loid) selection-induced ARGs were mainly associated with resistance-nodulation-division (RND) antibiotic efflux, which is distinct from what is observed in agricultural soil ecosystems. By using independent genome binning, metal(loid)s were shown impose coselection pressure on multiple ARGs residing on mobile genetic elements (MGEs), which promotes the dissemination of the antibiotic resistance. Interestingly, the current results showed that the density of several MGEs conferring ARGs was considerably higher in organisms most closely related to the priority pathogens *Pseudomonas aeruginosa* and *Escherichia coli*. Together, the results of this study indicate that mine soil ecosystems are important reservoirs of ARGs and that metal(loid)-induced coselection plays critical roles in the dissemination of ARGs in this type of soil habitat.

Key points

- Mining soil ecosystem is a reservoir of antibiotic resistance genes (ARGs).
- ARGs distribute via bacterial resistance-nodulation-division efflux systems.
- Metal(loid)s coselected ARGs residing on mobile genetic elements in *P. aeruginosa* and *E. coli*.

Keywords Metal(loid)s · Mine soil ecosystem · Shotgun metagenomic analysis · Antibiotic resistance gene · Metal resistance gene

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Introduction

The proliferation of antibiotic resistance genes (ARGs) is regarded as a growing public health concern worldwide (Berendonk et al. 2015; Zhao et al. 2018). Although much effort has been made to restrict antibiotic consumption worldwide, limited success has been achieved due to the lack of a comprehensive understanding of the occurrence and dissemination of ARGs in diverse environmental habitats (Berendonk et al. 2015). Soil ecosystems represent the largest microbial habitats on earth and are increasingly being identified as important reservoirs of ARGs available for exchange with clinical pathogens (Forsberg et al. 2014). A recent study conducted by Zhu et al. (2022) showed that soil ARGs could spread through the soil food web. However, the knowledge of the dissemination of ARGs in soil ecosystems is not well elaborated.

Recent evidence has shown that metal(loid)s could function as coselection agents in the proliferation of antibiotic resistance in soil ecosystems. For example, studies based on field-dependent approaches have demonstrated the coselection of metal and antibiotic resistance in soil habitats contaminated with a variety of metals (Baker-Austin et al. 2006; Guo et al. 2018). Furthermore, several attempts based on laboratory-dependent approaches have demonstrated that the selection of copper (Cu) (Berg et al. 2010) and nickel (Ni) (Hu et al. 2017) tends to increase the total number of ARGs in the agriculture soil ecosystem. More importantly, metal(loid)s are also reported to contribute to the dissemination of ARGs and their bacterial hosts via mobile genetic elements (MGEs), such as plasmids, integrons, and transposons, in soil systems (Berg et al. 2010; Ding et al. 2019; Hu et al. 2017). Considering that metal(loid)s cannot be biodegraded, it is of particular concern that metal(loid)s may exert longstanding coselection pressure for antibiotic resistance in soil ecosystems (Berg et al. 2010). However, the majority of these studies were conducted in agriculturally disturbed soil ecosystems, which generally exert not only metal(loid) but also antibiotic selection pressure on soil ARGs. Studies conducted in soil ecosystems that only exert metal(loid) selection pressure on soil ARGs are rare.

Mining is considered one of the most significant sources of metal(loid) contamination in soil ecosystems (Sun et al. 2018, 2020b). In contrast to agriculturally disturbed soil ecosystems, mine soil ecosystems lack anthropogenic sources of antibiotics, which may result in different soil ARG selection patterns between these two types of environmental habitats (Baker-Austin et al. 2006). Additionally, studies have shown that bacterial hosts may

play crucial roles in regulating the pattern of metal(loid)-induced coselection of ARGs in various habitats (Zhang et al. 2019). A study conducted by Shrestha et al. (2019) showed that the microbial composition was significantly different between agriculturally disturbed and mine soil ecosystems. As a result, it was proposed that the potential coselection pattern was different between agriculturally disturbed soil ecosystems and mine soil ecosystems. However, the current understanding of the metal(loid)-induced coselection of ARGs in mine soil ecosystems is poor. The limited examples of the dissemination of ARGs in mine soil ecosystems raise two questions: are there relationships between the distributional pattern of ARGs and metal(loid) selection in mine soil ecosystems? Are soil ARGs horizontally disseminated in mine soil ecosystems? The answers to these questions are essential for understanding the spread of ARGs in soil ecosystems.

In the current study, a mine area showing relatively low disturbance from agricultural activities was selected to investigate the impact of metal(loid) selection on the distributional pattern of ARGs. The objectives of this study were to (1) determine whether mine soil ecosystems are reservoirs of ARGs, (2) determine the coselection pattern of ARGs and metal resistance genes (MRGs), and (3) examine the dissemination of ARGs in mine soil ecosystems.

Materials and methods

Sampling sites

In the current study, a mine soil ecosystem located in Lanmuchang, Guizhou Province, southwestern China, was chosen to investigate the occurrence and dissemination of ARGs. At the sampling site, the soils close to the mine adit could be characterized as metal-rich soils compared to the soils at a greater distance from the adit; therefore, a distinct metal(loid) gradient was formed in this mine soil ecosystem. Three sampling sites (4 samples from each site) downstream of the mine area were selected; these sites were heavily (denoted as High, ZFSSA1-4), moderately (denoted as Middle, ZFSSB1-4), or weakly impacted (denoted as Low, ZFSSC1-4) by metal(loid)s. At each sampling site, a randomized field design was applied, and three pseudoreplicates (0–15-cm depth) of ~20 g of soil were obtained and mixed as a composite soil sample. All samples were transported to the laboratory and stored with ice packs (4 °C). Then, each sample was divided into two parts based on intended use: the portion to be used for DNA extraction was stored at –40 °C, and the second portion, for chemical analysis, was stored at 4 °C.

Chemical analysis

Soil samples were freeze-dried, and gravel, leaves, and plant roots were removed by passing through a 2-mm sieve before chemical analysis. By using a mortar and pestle, the samples were thoroughly ground before being passed through a 200-mesh sieve. The soil was then completely digested by using concentrated HF and HNO₃ (1:5, v/v) (Edgell 1989). Inductively coupled plasma mass spectrometry (ICP-MS) (Agilent, 7700x, Santa Clara, CA, USA) was used to measure the levels of metal(loid)s (Liu et al. 2020; Wang et al. 2021). The quality control method was described in our previous work (Xiao et al. 2021a). An elemental analyzer (vario MACRO cube, Elementar, Hanau, Germany) was employed to measure the total sulfur (TS), total carbon (TC), and total organic carbon (TOC) levels. For the purpose of soil pH measurement, a 10 g soil sample was mixed with 25 ml of distilled water in a 50-ml Erlenmeyer flask. Then, the soil pH was tested by using a calibrated HACH HQ30d pH meter (HACH, Loveland, USA).

Metagenomic sequencing and annotation

Total genomic DNA was extracted from 1 g of soil with the FastDNA® Spin Kit (MP Bio, Santa Ana, USA) by following the manufacturer's protocol. After extraction, the concentration and purity of the DNA were evaluated. The extracted DNA was then used for library construction and sequencing via a 101-bp paired-end strategy by using an Illumina PE150 at Novogene Bioinformatics Company (Beijing, China). After removing low-quality and ambiguous sequence reads, the paired-end sequence reads were merged into tags (average length of approximately 550 bp). The raw reads were deposited at NCBI under accession number PRJNA839862.

For ARG annotation, the merged tags of each sample were subjected to BLAST searches against the Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>) (e-value $\leq 1 \times 10^{-5}$) with Resistance Gene Identifier (RGI) software (Jia et al. 2017). The ARG sequences were then sorted into different types and subtypes following the protocol proposed by Luo et al. (2017). The abundances of different types and subtypes of ARGs were normalized according to reference sequence length (Luo et al. 2017). An ARG sequence was considered an MGE when its BLAST description included the keywords transposon, integrase, integron, and plasmid (Hu et al. 2017). For MRG annotation, sequences were subjected to BLAST searches against the metal resistance gene database (<http://bacmet.biomedicine.gu.se>). A sequence was considered an MRG when the following cutoffs were reached: e-value $\leq 1 \times 10^{-5}$ and similarity $\geq 80\%$ (Luo et al. 2017). Similar to the procedure for ARGs, the abundance of MRGs

was normalized according to reference sequence length. The taxonomy analysis of ARGs and MRGs was conducted with DIAMOND software (V0.9.9, <https://github.com/bbuchfink/diamond/>) with default settings (Hu et al. 2017).

Independent genome binning was used to explore the distributional pattern of ARGs and MRGs in the retrieved genomes from the mine soil samples. First, the trimmed reads from the paired samples were merged into new datasets for metagenomic assembly (Zhang et al. 2019). The merged reads were then mapped by using Bowtie2, and the coverage information for each sample is summarized in Supplemental Table S1. Thereafter, the contigs were used to construct genome bins by using MaxBin 2.0 (Wu et al. 2016) with the default parameters. Gene functions were predicted by annotating genome bins in seven databases, including the KEGG (Kyoto Encyclopedia of Genes and Genomes), GO (Gene Ontology), and NR (Non-Redundant Protein) databases.

Data analysis

Principal component analysis (PCoA) and Procrustes analysis were performed, and heatmaps of the relative abundances of ARGs and MRGs were generated by using R (v.2.13.1; <http://www.r-project.org/>) with the vegan, igraph, and Hmisc packages (Luo et al. 2017). To test the correlation between the ARG composition and environmental variables, canonical correspondence analysis (CCA) was conducted using CANOCO 5.0 software (ter Braak and Smilauer 2012). Furthermore, Spearman's correlation analysis was used to test the relationship between environmental parameters and various ARGs.

Results

Antibiotic resistome characterization in the mine soil ecosystem

In the current study, a total of 12 soil samples were collected along a metal(loid) gradient to perform a shotgun metagenomic analysis of ARGs. In total, approximately 168,948.95 Mbp of clean data were obtained by paired-end sequencing (Supplemental Table S1). The total number of genes increased markedly from 6121 ± 250 in weakly metal(loid)s impacted samples (Low group) to 9322 ± 523 in the heavily metal(loid)s impacted samples (High group) (Supplemental Table S1). Furthermore, the number of ARGs increased markedly from 271 ± 17 in the Low group to 358 ± 32 in the Middle group and then decreased to 309 ± 22 in the High group (Supplemental Table S1). A total of 819 ARG subtypes were detected in all the soil samples. The detected ARGs were associated with ten major antibiotic families

but were predominantly associated with resistance-nodulation-division (RND) antibiotic efflux, ATP-binding cassette (ABC) antibiotic efflux, β -lactamase activity, and major facilitator superfamily (MFS) antibiotic efflux, accounting for 60.02–73.94% of the total ARGs (Fig. 1A). The main resistance mechanisms across the soil samples were most often related to antibiotic efflux and antibiotic degradation, followed by antibiotic inactivation (Fig. 1A). These ARG types mainly included the fluoroquinolone, macrolide, aminoglycoside, cephalosporin, and tetracycline resistance genes (Fig. 1B). The 10 most abundant ARG subtypes accounted for 21% of the total ARG coverage and are summarized in Supplemental Table S2.

The ARG composition is shifted by metal(loid)s

The PCoA results showed that the soil samples were clustered into three groups, which was consistent with the distributional pattern of metal(loid)s (Fig. 2B). This result suggested that the ARG composition differed greatly among samples from different areas of the metal(loid) gradient. Accordingly, as shown in Supplemental Fig. S1, the top 50 most abundant ARGs were clearly clustered into three categories under the selection pressure imposed by metal(loid) contamination. Then, CCA was used to determine the contribution of the metal(loid)s to influence the ARG composition (Fig. 2D). The results showed that metal(loid)s, including Cu, Ni, Zn, As, and Hg, were considered as important environmental parameters influencing the distributional pattern of soil ARGs according to the length corresponding to these metal(loid)s. In addition, Spearman's correlation analysis was used to show the strong relationship between the metal(loid) content and the relative abundance of ARGs (family level) (Fig. 2C). To further identify the distributional pattern of soil resistance among metal(loid) gradients, a linear model analysis was conducted to identify soil ARGs that were significantly enriched in the High, Middle, and Low groups (Fig. 2A). With this approach, three distinct soil ARG subcommunities thriving along the metal(loid)

gradient were identified (Fig. 2A). The subcommunity designated Low group showed significant enrichment of *NPS-1*, *ErmE*, *nalD*, *cmlA4*, and *emeA*, which mainly affiliated to β -lactamase. The subcommunity designated Middle group showed significant enrichment of *tet36*, *SMB-1*, *OXA-118*, *rphA*, and *smeC*, which mainly affiliated to β -lactamase. The subcommunity designated High group showed significant enrichment of *OXA-361*, *mdtA*, *marR*, *LRA-5*, and *vanXYG*, which mainly affiliated to β -lactamase and RND antibiotic efflux system (Fig. 2A).

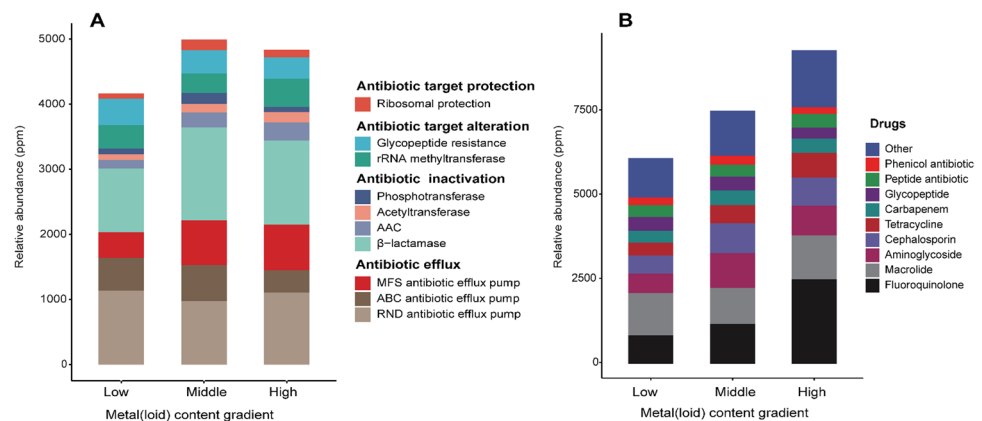
Metal(loid)s coselect ARGs in mine soil ecosystem

Procrustes analysis showed a strong correlation between the ARG profile and taxonomic groups ($M^2 = 0.5339$, $p = 0.004$) (Fig. 3B). *Streptomyces*, *Rhodanobacter*, *Pseudomonas*, *Phenylobacterium*, *Lysobacter*, *Caulobacter*, and *Bradyrhizobium* were the major hosts of these combinations of ARGs and MRGs (Fig. 4). In addition, the results showed that in the bacterial host, there was coselection for resistance genes related to antibiotics and metal(loid)s (Fig. 4). For example, the major bacterial hosts of the ARGs associated with RND antibiotic efflux were coselected with genes for cadmium and zinc resistance. Moreover, the results showed that ARGs related to RND antibiotic efflux were strongly associated with As MRGs (i.e., arsenate reductase, arsenite *S*-adenosylmethyltransferase, and arsenic resistance genes) and Hg MRGs (i.e., mercuric reductase, mercuric ion transporter, and mercury transcriptional regulator genes) (Fig. 4). Notably, the bacterial hosts of the combination of β -lactam ARGs and MRGs were markedly distinct from the hosts of the combination of RND antibiotic efflux genes and MRGs (Fig. 4).

Colocalization between ARGs and MGEs

MGEs, including plasmids, conjugative transposons, insertion sequences (ISs), and integrons, were identified as key factors in the movement and acquisition of ARGs from

Fig. 1 Changes in the relative abundance (RPM, reads per million mapped reads) of (A) detected ARG types and (B) drug compositions across the metal(loid) content gradient (Low, Middle, and High) in the mine soil ecosystem



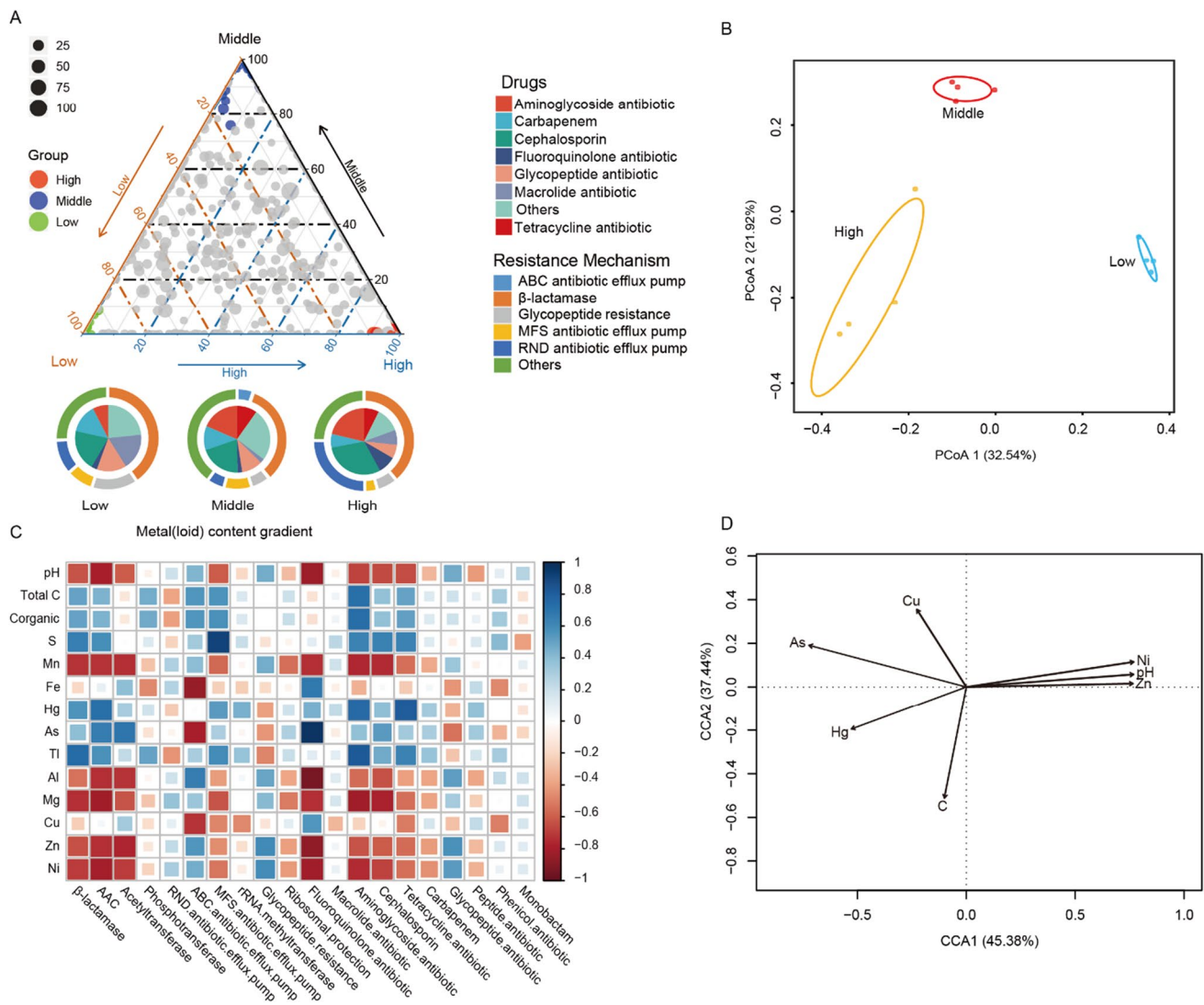


Fig. 2 (A) Distribution pattern of ARG subtypes, resistance mechanism composition (outer circle), and drug composition (inner circle) across the metal(loid) content gradient in the mine soil ecosystem; The detailed information of ARG subtypes was listed in Supplementary Table S9; (B) PCoA analysis revealed the distribution pattern of

ARGs subtypes across metal(loid) content gradient; (C) the Spearman's correlation between ARG types and environmental factors; (D) CCA analysis reveals the impacts of environmental factors to the distribution of ARG types

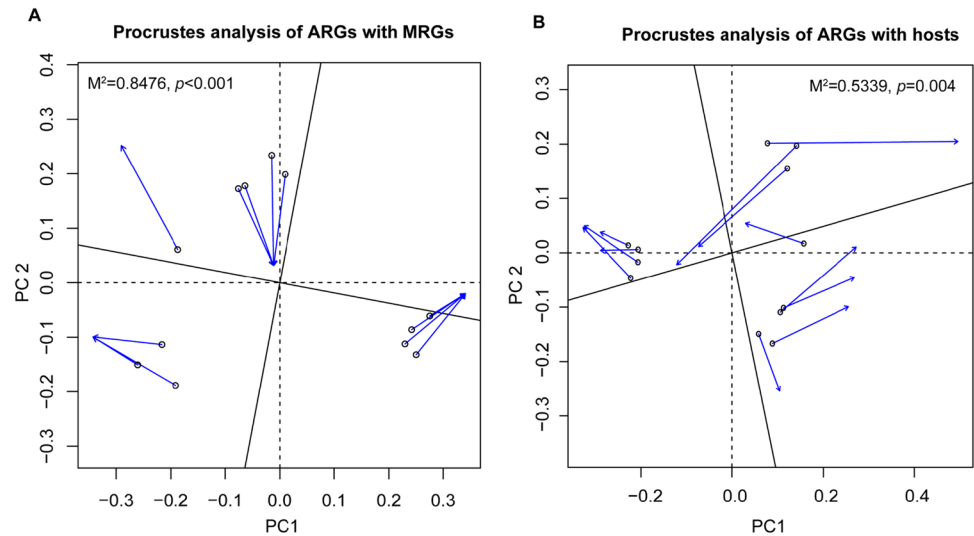
various microorganisms via the horizontal gene transfer pathway. In the current study, MGEs were found to be present across soil ARGs. In total, 65 ARGs were associated with MGEs, including 35 plasmids, 28 integrons, and 2 ISs. Moreover, as shown in Supplemental Fig. S2, the distributional pattern of MGEs was characterized by clear clustering into three categories under the selection pressure of metal(loid)s. Information about the MGE composition is summarized in Supplemental Table S3. Generally, 65 ARGs carried MGEs, and 4 ARGs carried multiple MGEs (≥ 2 MGEs). In this study, the MGE-carrying ARGs were mainly affiliated with RND antibiotic efflux pumps (*adeF* and *lmrD*) and β -lactamases (*VIM-29*, *TEM-157*, *TEM-220*,

and *OXA-192*) (Fig. 5). MGE-carrying ARGs were carried by bacterial species associated with *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Burkholderia* sp., *Azospirillum* sp., *Ralstonia solanacearum*, and *Salmonella enterica* (Fig. 5).

Coselection of ARGs and MRGs in assembled genome bins

Here, sequence composition-independent binning was used to explore the coselection of ARGs in mine soil samples. Four ARG-carrying genome bins were successfully retrieved from metagenome datasets. The annotation information

Fig. 3 Procrustes analyses revealed the correlation between (A) ARG subtypes and MRGs and (B) ARG subtypes and microbial community (OTU level) based on Bray–Curtis results of their abundance



for each bin is summarized in Supplemental Table S4. The total length of the four bins ranged from 3,271,026 to 4,963,481 bp (Supplemental Table S4). The percentage of ARG numbers for each bin ranged from 1.2 to 4% (Supplemental Table S4). In addition, binning analysis showed the colocalization of ARGs, MRGs, and MGEs in the soil samples. The results showed that bin 147 carried 1 ARG, 16 MRGs, and 2 transposon PSIs (Supplemental Fig. S3, detailed information in Supplemental Table S5). Bin 155 carried 4 ARGs, 1 MGE, 27 MRGs, and 3 transposon PSIs (Supplemental Fig. S3, detailed information in Supplemental Table S6). Bin 156 carried 6 ARGs, 2 MGEs, 19 MRGs, and 3 transposon PSIs (Supplemental Fig. S3, detailed information in Supplemental Table S7). Bin 157 carried 1 ARG, 17 MRGs, and 2 transposon PSIs (Supplemental Fig. S3, detailed information in Supplemental Table S8).

Discussion

The occurrence of soil ARGs in the mining soil ecosystem

The current study identified a total of 819 ARG subtypes in all the samples, showing that mine soil ecosystems are important reservoirs of ARGs. Among these ARGs, most were associated with the fluoroquinolone and macrolide antibiotic classes (Fig. 1B). Generally, the ARG types associated with fluoroquinolone and macrolide antibiotics in soil ecosystems are related to the extensive use of the corresponding antibiotics associated with human activities (Luo et al. 2017). However, the current study site is a naturally occurring metal-rich site with relatively low disturbance, as reported previously (Xiao et al. 2004), suggesting that direct selection exerted by antibiotics might not be a key stressor

shaping the soil ARG composition at this site. Importantly, the results of this study showed that the distribution of these ARGs was likely shaped by the selective pressure exerted by metal(loid)s. For example, the ARG profiles were clearly clustered into three categories under the selection pressure imposed by the metal(loid) gradient (Fig. 2B and Supplemental Fig. S2). Furthermore, the statistical correlation analysis (Fig. 2C) and CCA (Fig. 2D) demonstrated that metal(loid)s have profound impacts on the distribution of ARGs. These results corroborate previous research showing that metal(loid) selection had significant effects on the distribution of soil ARGs. For example, Poole (2017) showed a positive relationship between antibiotic resistance and copper and zinc levels. A study conducted by Hu et al. (2017) also showed that Ni has profound impacts on the distributional pattern of ARGs (Hu et al. 2017). Furthermore, Zeng et al. (2010) identified the prevalent proliferation of *merT* and *merR* in surface soils with elevated mercury levels.

Prior studies have shown that microbial taxa carry specific (Forsberg et al. 2014) and drive similar abundance trends among different samples (Li et al. 2015). In this study, Procrustes analyses showed that the ARG composition was significantly correlated with the distribution of bacterial hosts ($p = 0.004$) (Fig. 3B). In general, elevated metal(loid) levels increase the abundance of ARGs through the enrichment of metal(loid)-resistant bacteria (Zhang et al. 2019). The results of this study showed that the core ARGs were mainly harbored by hosts from the genera *Streptomyces*, *Rhodanobacter*, *Pseudomonas*, *Phenylobacterium*, *Lyso-bacter*, *Caulobacter*, and *Bradyrhizobium* (Fig. 4), which were previously shown to be relatively tolerant to metal(loid) stress and to constitute the predominant members of the soil microbiome in mine areas (Sun et al. 2020a, b; Xiao et al. 2021b). These findings are consistent with the previous finding that metal(loid)-resistant bacteria carrying

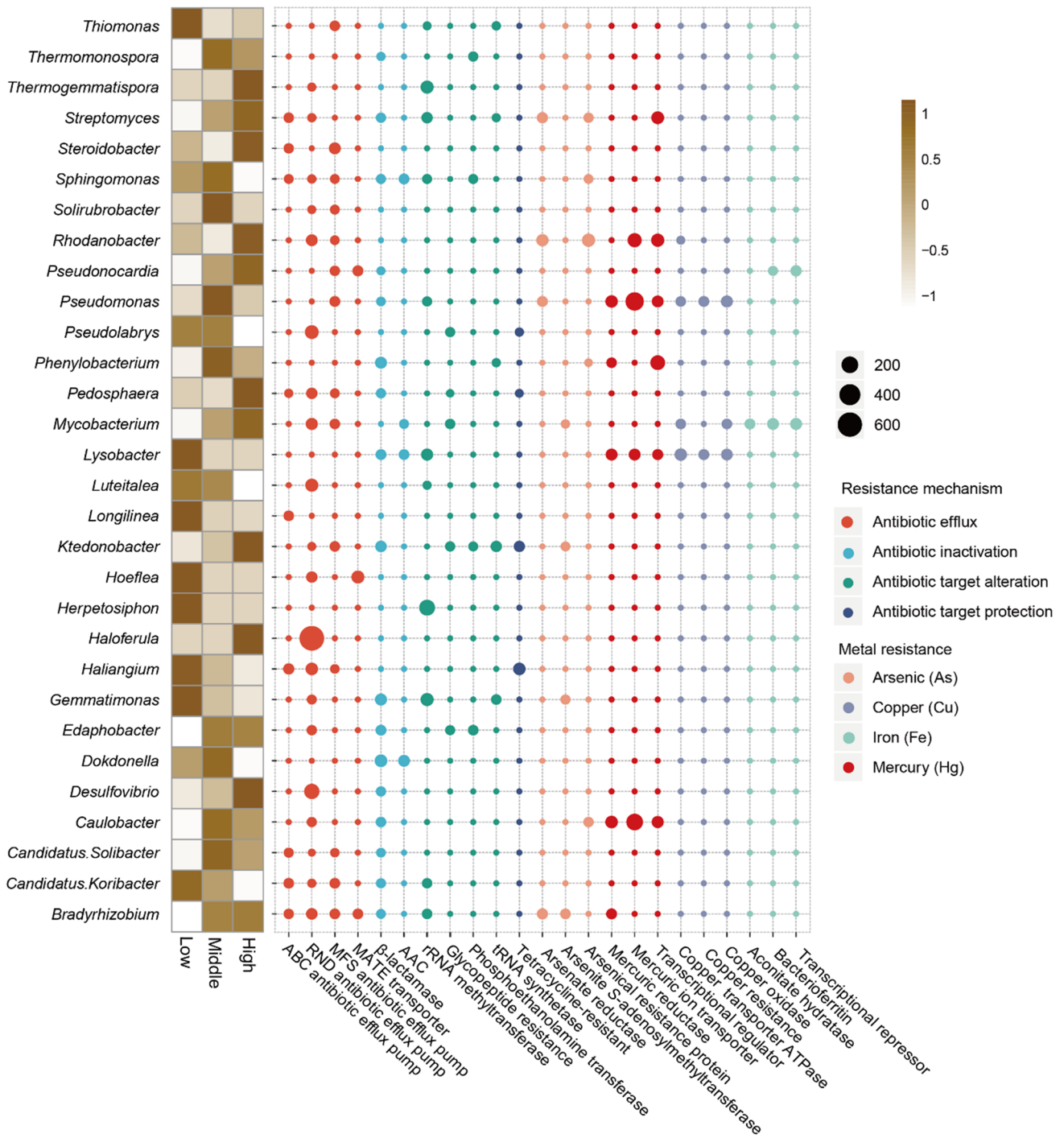


Fig. 4 The relative abundance of the major potential hosts (genera with a percentage of over 1% in any sample) for ARG and MRG types across the metal(loid) content gradient. The size of the circle is proportional to the percentage of the relative abundance of bacterial hosts

multiple ARGs are much more tolerant to antibiotic stress than metal(loid)-sensitive bacteria (Baker-Austin et al. 2006). Taken together, the results of this study provide evidence that mine soil ecosystems are an important reservoir of ARGs and that the distributions of these ARGs are mainly determined by metal(loid) selection.

Metal(loid)s coselect ARGs via the RND antibiotic efflux system

Metal(loid)-induced coselection of antibiotic resistance has been observed in several heavy metal-contaminated agricultural soils (Berg et al. 2010). These metal(loid)-coselected

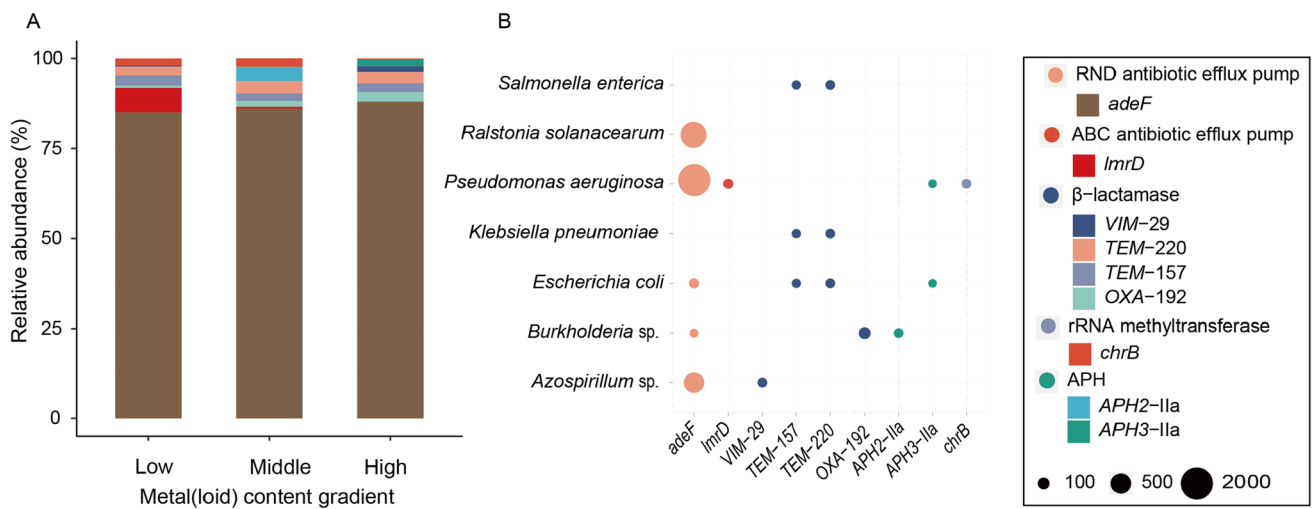


Fig. 5 (A) Distribution pattern of the ARGs carrying mobile genetic elements (MGEs) across the metal(loid) content gradient in the mine soil ecosystem; (B) the relative abundance of MGEs carrying ARGs

in their putative hosts. The size of the circle is proportional to the percentage of the relative abundance of bacterial hosts

ARGs mainly contribute to coresistance via the prevalence distribution of antibiotic agents and metal(loid)s (Seiler and Berendonk 2012). In contrast, metal(loid) selection-induced ARGs were mainly associated with RND antibiotic efflux in this study (Figs. 1A and 2C). This result corroborates a prior study showing that the relative abundance of ARGs affiliated with RND antibiotic efflux was increased after metal(loid) contamination (Otakuye et al. 2010). ARGs affiliated with RND efflux pumps play established roles in the detoxification of intracellular metabolites and intrinsic and acquired resistance (Otakuye et al. 2010; Tseng et al. 1999). The current study showed that ARGs affiliated with RND efflux pumps were significantly correlated with metal(loid) content (Supplemental Fig. S4). Furthermore, metal(loid)s were shown to play an important role in maintaining the stability of the cooccurrence network of ARGs affiliated with RND efflux pumps (Supplemental Fig. S5). These findings are partially supported by prior studies showing that the RND efflux system exports a wide range of metal(loid)s, including Cu(I), Ag(I), Zn(II), Ni(II), Co(II), and Cd(II) (Baker-Austin et al. 2006). Therefore, it is fair to propose that metal(loid)-induced bacterial RND antibiotic efflux systems play critical roles in the coselection of ARGs in mine soil ecosystems. Recent evidence has shown that metal(loid)s share similar pathways of action as antibiotics, which can lead to coregulation, contributing to the emergence of antibiotic resistance in microbial taxa via RND efflux pumps (Delmar et al. 2014). Consistent with this, Procrustes analyses showed a significant correlation between the ARG affiliated with RND efflux pumps and MRG compositions ($p < 0.001$) (Fig. 3A).

Furthermore, through metagenomic contig assemblage, the study showed that ARGs affiliated with the RND

antibiotic efflux system co-occurred with MRGs in the assembled bins. For example, *acrA/B*, affiliated with the RND efflux system, was closely associated with *copB* in bin_155 and bin_156 (Fig. 6A and Supplemental Fig. S4). The *copB* gene is associated with the Cu-ATPase pump (Barahona et al. 2020) and frequently co-occurs with genes affiliated with the RND system, thereby increasing the tolerance of the host to grow in the presence of high Cu concentrations (González et al. 2014; Tangjian et al. 2017). The *acrA* and *acrB* genes are important components of the RND efflux system (Paolo et al. 2013; Pos 2009) and play a major role in actively recognizing and expelling diverse antibiotics from the interior of bacterial cells (Piddock 2006; Tseng et al. 1999). Notably, the abundances of *copB* were positively correlated with that of Mg, Zn, and Ni (Fig. 7). These results suggest that metal(loid) stress may trigger *copB* and result in the coselection of *acrA* and *acrB* affiliated with the RND efflux system. Similarly, the results obtained by Rodrigues et al. (2008) showed that *copB* was colocalized with *acrA/B* in the genome of *Xylella fastidiosa* isolated from plant samples (Rodrigues et al. 2008). However, this gene combination is rare in mine soil ecosystems. Furthermore, the *copB* gene was shown to be colocalized with *mexE* and *mexF*, members of the RND efflux system, in bin_155 and bin_156 (Supplemental Fig. S3). However, this gene combination has not been previously reported, suggesting that this combination represents a new combination. Other gene combinations between ARGs affiliated with the RND efflux system and MRGs were also identified in the current study. For example, *mprF* and *mexK*, which are important members of the RND efflux system (Dey and Wall 2014), were positioned close together with the mercury resistance

Fig. 6 Arrangements of ARGs, MRGs, and mobile genetic elements (MGEs) in assembled (A) bin 156-scaffold 9 and (B) bin 156-scaffold 12

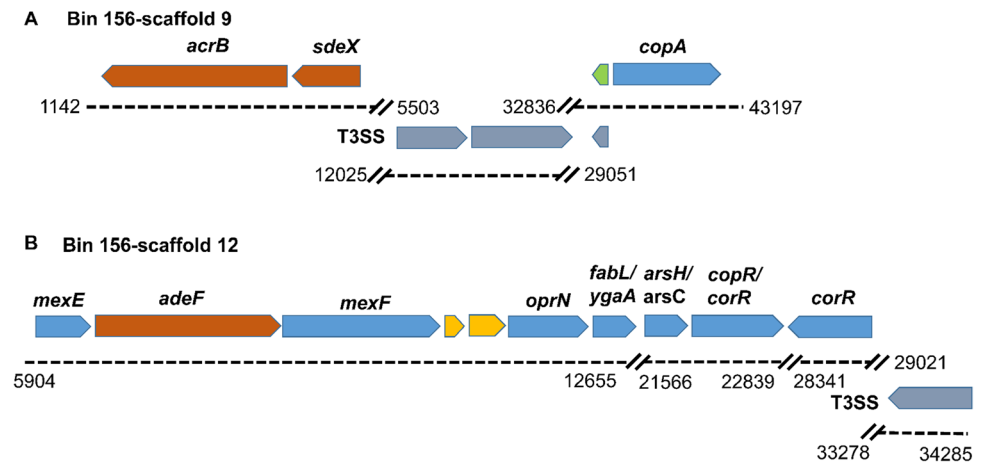
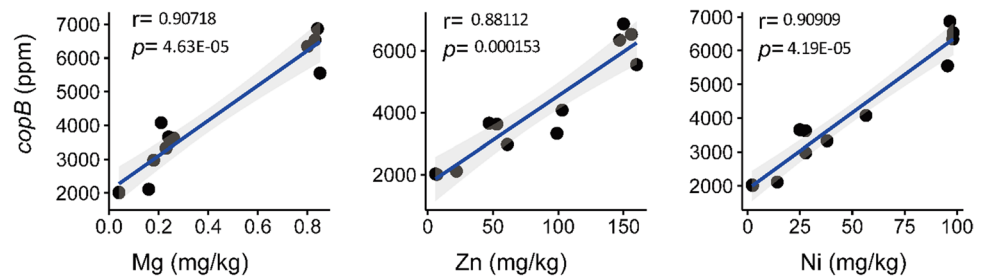


Fig. 7 Spearman's correlation between the content of Mg, Zn, and Ni and the relative abundance of *copB*



regulator *merE* in the assembled bin_155. These findings suggested that metal(loid) transcriptional regulators are likely coselected with ARGs affiliated with RND antibiotic efflux. Taken together, the results of this study provide evidence that metal(loid) contamination can lead to coselection between MRGs and ARGs associated with RND antibiotic efflux systems in mine soil ecosystems.

The dissemination and environmental risk of soil ARGs

The ability of soil bacteria to horizontally disseminate ARGs via MGEs is of considerable public health concern (Hu et al. 2017). Importantly, the current study showed diverse MRGs colocalized with MGEs carrying ARGs affiliated with RND antibiotic efflux systems in the assembly bins (Figs. 6 and Supplemental Fig. S4). For example, *corR* and *mexF* colocalized with an MGE in assembly bin_155. The colocalization of the gene combination of *merE* and *mexK* with an MGE was detected in assembly bin_155. The *oprN* gene co-occurred with *mexF* and colocalized with the same MGE in assembly bin_156. These results suggest that metal(loid)-induced coselection facilitates the dissemination of ARGs associated with RND antibiotic efflux systems via MGEs in mine soil ecosystems, supporting the previously reported interrelationships between ARGs associated with RND antibiotic efflux systems and MGEs in soil ecosystems (Guo

et al. 2018; Hu et al. 2017). In general, the presence of ARGs carrying MGEs was strongly related to metal(loid) levels (Peng et al. 2021). Consistent with this, the numbers of these associations between ARGs affiliated with RND antibiotic efflux systems and MGEs were higher in samples with high levels of metal(loid)s (Supplemental Fig. S2). A prior study demonstrated that bacterial hosts were the dominant determinant for the spread of ARGs via MGEs. Interestingly, the current study showed that the density of 4 MGEs conferring ARGs was considerably higher in organisms that were most closely related to *P. aeruginosa* and *E. coli* (Fig. 5). *P. aeruginosa* is considered a priority pathogen by the World Health Organization (WHO) and is commonly resistant to several antibiotics (Botelho et al. 2019). *E. coli* has been recently recognized as a global pathogen and leads to bloodstream infections and urinary tract infections in hospitals (Makrina et al. 2011). *P. aeruginosa* and *E. coli* are widely detected in agricultural soils contaminated by feces (Milton et al. 2018; Dusek et al. 2019). Despite their low abundance, *P. aeruginosa* and *E. coli* are also detected in metal(loid) mining site and are tolerant of elevated content of metal(loid)s (Arkoc et al. 2016; Ayansina et al. 2019). These facts suggest that *P. aeruginosa* and *E. coli* were commonly resistant to antibiotics as well as to metal(loid)s, which is consistent with prior studies. For example, *P. aeruginosa* was reported to be resistant to cadmium and correlated with chloramphenicol and streptomycin (Deredjian et al. 2011). In addition,

E. coli is resistant to zinc and frequently associated with resistance to ampicillin, nalidixic acid, and tetracycline. Thus, this raises the possibility that the MGEs conferring ARGs may be a reservoir of resistance genes available for exchanging with clinical pathogens. Taken together, these results suggested that elevated metal(loid) content imposes selection pressure on multiple ARGs associated with RND antibiotic efflux systems residing on MGEs, thereby promoting the dissemination of the antibiotic resistome in mine soil ecosystems.

The proliferation of ARGs is regarded as a growing public health concern worldwide. In the current study, using shotgun metagenomic analysis, mine soil ecosystems were identified as important pool of ARGs. Metal(loid)s were shown to impose selection pressure on the occurrence and dissemination of ARGs in mine soil ecosystems. Furthermore, the results showed that metal(loid) contamination can lead to coselection between MRGs and ARGs associated with RND antibiotic efflux systems. By using independent genome binning, the results showed that the density of several MGEs conferring ARGs was considerably higher in the priority pathogens *P. aeruginosa* and *E. coli*. Given that mine soil ecosystems are the most significant sources of metal(loid) contamination and that the levels of metal(loid)s in these ecosystems are frequently several orders of magnitude greater than those in other natural ecosystems, mine soil ecosystems are proposed to be nonnegligible environmental habitats that facilitate the occurrence and dissemination of soil ARGs.

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Author contribution EZX and TFX contributed to the study conception and design. Material preparation, data collection, and analysis were performed by EZX, WMS, and ZPN. The first draft of the manuscript was written by EZX; YQW, JMD, WJF, FDM, and TFX commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests The authors declare no competing interests.

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