Sub-inhibitory concentrations of heavy metals facilitate the horizontal transfer of plasmid-mediated antibiotic resistance genes in water environment*

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ABSTRACT

Although widespread antibiotic resistance has been mostly attributed to the selective pressure generated by overuse and misuse of antibiotics, recent growing evidence suggests that chemicals other than antibiotics, such as certain metals, can also select and stimulate antibiotic resistance via both co-resistance and cross-resistance mechanisms. For instance, tetL, merE, and oprD genes are resistant to both antibiotics and metals. However, the potential de novo resistance induced by heavy metals at environmentally-relevant low concentrations (much below the minimum inhibitory concentrations [MICs], also referred as sub-inhibitory) has hardly been explored. This study investigated and revealed that heavy metals, namely Cu(II), Ag(I), Cr(VI), and Zn(II), at environmentally-relevant and sub-inhibitory concentrations, promoted conjugative transfer of antibiotic resistance genes (ARGs) between E. coli strains. The mechanisms of this phenomenon were further explored, which involved intracellular reactive oxygen species (ROS) formation, SOS response, increased cell membrane permeability, and altered expression of conjugation-relevant genes. These findings suggest that sub-inhibitory levels of heavy metals that widely present in various environments contribute to the resistance phenomena via facilitating horizontal transfer of ARGs. This study provides evidence from multiple aspects implicating the ecological effect of low levels of heavy metals on antibiotic resistance dissemination and highlights the urgency of strengthening efficacious policy and technology to control metal pollutants in the environments.

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1. Introduction

Widespread antibiotic resistance poses a serious threat to human health because it is associated with the loss of therapeutic potential for antibiotics and consequent morbidity and mortality (Allen et al., 2010; Levy and Marshall, 2004; Ashbolt et al., 2013). The observed increase in antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in both clinical and natural environments has been attributed to the selective pressure generated by overuse and misuse of antibiotics in medicine (Ashbolt et al., 2013; Huang et al., 2012), veterinary animal feeding (Berendonk et al., 2015; He et al., 2016), and aquaculture (Berendonk et al., 2015). Recently, growing evidence suggests that chemicals other than antibiotics can also select and stimulate antibiotic resistance, and they include heavy metals (Baker-Austin et al., 2006; Seiler and Berendonk, 2012), disinfectants (Guo et al., 2015; Zhang et al., 2017), disinfection by-products (Lv et al., 2015; Li et al., 2016), and nano-materials (Qiu et al., 2012; Ding et al., 2016).

Heavy metals exist naturally in the environment, and anthropogenic activities significantly accelerate the release and accumulation of metals in various environments (Seiler and Berendonk, 2012; Rodríguez Martín et al., 2015; Zhang et al., 2015; Wang et al., 2015a,b). A limited number of studies have reported the roles of heavy metals in the co-selection of antibiotic resistance in freshwater (Stepanauskas et al., 2006), soils (Cabral et al., 2016), and animal manures (Zhu et al., 2013). The co-selection...
mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to both antibiotics and metals) (Baker-Austin et al., 2006; Seiler and Berendonk, 2012). Genes involved in metal and antibiotic resistances are generally located on mobile or mobilizable genetic elements, such as plasmids, transposons, and integrons, which may be transferred between microbial communities (Suzuki et al., 2012; Andersson and Hughes, 2014).

The acquisition and spread of antibiotic resistance have been enhanced by the recruitment of antibiotic resistance genes (ARGs) into bacteria via de novo mutation (Andersson and Hughes, 2014) and/or horizontal transfer of mobile genetic elements (MGEs) (Ashbolt et al., 2013; Berendonk et al., 2015; Andersson and Hughes, 2014), including transposons, integrons and plasmids. Particularly, the horizontal transfer of ARGs is considered an important driver for acquiring and spreading ARGs in various environments (Ashbolt et al., 2013; Berendonk et al., 2015; Andersson and Hughes, 2014; Martinez et al., 2015). A few studies have investigated the impact of metal ions on the horizontal transfer of ARGs and revealed that Cu(II), Zn(II), and Cd(II), at concentrations above minimum inhibitory concentrations (MICs), and showed decrease in the efficiencies of conjugative transfer (Suzuki et al., 2012; Martinez et al., 2006). However, metals (e.g., Cu, Zn, Cd, Cr, Ph, Ag, Hg) in soil (Teng et al., 2014; GB15618–2008), water (Waseem et al., 2014; GB3838–2002), animal manure (Zhu et al., 2013) and gut microenvironments (Breton et al., 2013) are usually present at sub-inhibitory concentrations (below MICs, also referred to as sub-lethal levels) (Seiler and Berendonk, 2012; Nies, 1999). Evidences showed that the horizontal transfer of ARGs between bacteria can be promoted by sub-inhibitory levels of antibiotics and disinfectants (Zhang et al., 2017; Baharoglu et al., 2013). How these heavy metals, at environmentally relevant and sub-inhibitory levels, affect the conjugative transfer of ARGs has rarely been investigated (Suzuki et al., 2012; Martinez et al., 2006).

Recent research has revealed that the molecular mechanisms for stimulating the horizontal transfer of ARGs by sub-inhibitory levels of antibiotics and disinfectants display both similarity and differences from those known at above-MIC levels, and particularly involve reactive oxygen species (ROS) response systems (Beaber et al., 2004; Baharoglu et al., 2013) and the SOS response pathway (a global response to DNA damage in which the cell cycle is arrested and DNA repair and mutagenesis are induced) (Andersson and Hughes, 2014; Beaber et al., 2004). Previous studies have shown that certain heavy metal ions, such as Cu(II), Ag(I), Cr(VI), and Zn(II), can induce oxidative stress and genotoxicity (Lemire et al., 2013; Asakura et al., 2009). Therefore, we hypothesized that these heavy metals, at sub-inhibitory and environmentally relevant concentrations, can promote the horizontal transfer of ARGs. To test this hypothesis, we evaluated the effect of these metals, at sub-inhibitory concentrations, on the acceleration of the plasmid-mediated horizontal transfer of ARGs. Furthermore, we probed the involved mechanisms promoting horizontal transfer, which included the formation of intracellular ROS, cell membrane permeability, and altered expression levels of genes involved in the SOS response, oxidative stress, and conjugative transfer. To the best of our knowledge, this is the first study to investigate the effects and mechanisms of sub-inhibitory levels of metal ions on the horizontal transfer of ARGs. This study provides novel understanding on the environmentally-relevant low levels of heavy metals on the dissemination of antibiotic resistance, and the results are of great importance for evaluating heavy metal pollutants, controlling antibiotic-independent resistance, and assessing heavy metal-induced health risks.

2. Material and methods

2.1. Bacterial strains, plasmids, and culture conditions

As the most widely used model microorganism, Escherichia coli (E. coli) was selected to evaluate the efficiency of conjugative transfer. The donor E. coli S17–1 harbors a mobilizable plasmid pCM184–Cm (7625 bp), which is regulated by RP4 DNA segments in the host’s chromosome (Zhang et al., 2017). The plasmid pCM184–Cm carries the resistance genes to ampicillin (Amp), tetracycline (Tet), and chloramphenicol (Chl) (Chen et al., 2015; Zhang et al., 2017). The E. coli K12 MG1655, containing the pUA139 plasmid that carries the kanamycin (Km) resistance gene, was employed as the recipient strain. Both the donor and recipient strains were incubated at 37 °C in Luria broth (LB) medium (10 g tryptone, 5 g yeast extract and 10 g sodium chloride in 1 L deionized water; pH: 7.4) supplemented with 20 mg/L Chl and 100 mg/L Km, and shaken at 180 rpm for 16–18 h. Then, the prepared bacterial strains were applied to a sub-inhibitory concentrations determination, conjugation experiments, and mechanistic investigations in the following experiments.

2.2. Evaluation of the impact of heavy metals on conjugative transfer rates of ARGs

In this study, we used the optimized conjugation model to evaluate the conjugative transfer efficiencies of ARGs between two E. coli strains, according to a previous study in our lab (Zhang et al., 2017). Briefly, the overnight cultures of donor and recipient strains were centrifuged at 8000 × g (Centrifuge 5424/5424 R, Eppendorf, Hamburg, Germany) for 4 min and washed with phosphate buffered saline (PBS) buffer. Then, the bacteria were re-suspended in PBS buffer to a concentration of 10^8–10^9 CFU/mL, and the donor and recipient were mixed at a ratio of 1:5:1. Then, the bacterial mixtures were treated with different metal ions (see Section 2.3) at 37 °C for 4 h. The samples without metal treatment and incubated in PBS buffer were used as a control group.

Then mixtures were appropriately diluted in PBS and plated on LB agar plates containing the appropriate antibiotics to determine and calculate the numbers of donors, recipients, and transconjugants as described previously (Zhang et al., 2017). Finally, LB agar plates containing 20 mg/L Chl and 100 mg/L Km were used to isolate, verify and quantify the transconjugants from the mixed bacterial cultures, and the recipient concentrations treated with various concentrations of heavy metals were determined by using LB agar plates containing 100 mg/L Km. The efficiency of conjugative transfer is presented as the numbers of transconjugants per recipient cell. At least three parallel conjugation experiments were processed.

2.3. Minimum inhibitory concentrations (MICs) analysis

The MICs were determined to estimate the antimicrobial activity of each heavy metal ion against E. coli K12 according to previous studies (Li et al., 2016; CLSI, 2012). Briefly, the overnight culture of E. coli K12 was 1:100 diluted to the initial cell density of about 10^6 CFU/mL, and then 5 μL of selected E. coli K12 cultures, 15 μL of serially two-fold diluted heavy metals as well as 130 μL of fresh LB broth were introduced into each 150 μL well of 96-well microplates. Sterilized PBS (pH: 7.4) was set as blank control. Compounds in present study, including CuSO4·5H2O, Ag2SO4·3H2O, K2Cr2O7 and ZnSO4·7H2O were purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). After overnight incubation (16–18 h) at 37 °C, the optical density at 600 nm (OD600) was measured by a microplate spectrophotometer (SynergyH1,
Synergy HTMulti-Mode, Biotech, Winooski, VT, USA). MICs were calculated as the concentrations of metal ions that caused 90% growth inhibition of the original bacterial cells. Each test was carried out at least in triplicate.

2.4. Analysis of intracellular ROS production

Intracellular ROS production in *E. coli* exposed to metal ions was determined by using fluorescence probe 2′, 7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Saint Louis, MO, USA) as described previously (Zhang et al., 2017). Briefly, the bacterial cultures were washed and re-suspended in PBS at approximately 10⁶ CFU/mL. Then the DCFH-DA probe was added at a final concentration of 10 μM, and incubated in the dark for 20 min at 37 °C with gentle shaking. After removing extracellular DCFH-DA probe by washing with PBS, bacterial cells were treated with metals as described above. Then, all the metal-treated samples and controls (without metal treatment) were transferred into a 96-well plate (200 μL per well) for fluorescence intensity (FI) measurement (488 nm excitation, 525 nm emission) using a microplate reader (Synergy H1, Synergy HTMulti-Mode, Biotech, Winooski, VT, USA). All metal-induced ROS levels are presented as FI ratios of experimental groups vs. controls. All experiments were performed in triplicate.

In parallel, to confirm the role of ROS in the conjugation process and ARG transfers, a ROS scavenging experiment was conducted using thiourea (CH₄N₂S, TU) (TCI, Tokyo, Japan), a scavenger of ROS. In this experiment, thiourea (at a final concentration of 100 mM) was added to each bacterial culture together with metals. Then, the conjugative transfer rates in presence of a ROS scavenger were compared to experiments without the scavenger.

2.5. Analysis of cell membrane permeability using a flow cytometer (FCM)

The cell membrane permeability of donor and recipient bacteria treated with metal ions were evaluated by a FCM (BD Biosciences, San Jose, CA, USA) and propidium iodide (PI) (Otto et al., 2010), and the detailed protocols and data analysis are presented in Text S1.

2.6. Transcriptomic analysis of the oxidative stress, SOS response, and the lipid-related pathway

A number of green fluorescent protein (GFP) transcriptional fusions were constructed in *E. coli* K12 MG1655 (Open Biosystem, Huntsville, AL, USA) and used to evaluate the transcriptomic effects of metal ions, according to previous studies in our group (Zhang et al., 2017; Li et al., 2016). The genes detected in the present study are summarized in Table S1, and the detailed protocols are presented in Text S2.

2.7. Detection of mRNA expression by quantitative reverse transcription PCR (qRT-PCR)

A qRT-PCR assay was employed to evaluate the transcription of genes associated with horizontal transfer during conjugative processes that were potentially impacted by metal ions (Zhang et al., 2017; Qiu et al., 2012; Wang et al., 2015a). Nine target genes,
including outer membrane protein genes (ompA and ompC), conjuga-
tive transfer-related genes involved in global regulator genes of
horizontal transfer (korA, korB, and trbA), mating pair formation
(Mpf) system genes (trbBp and traF), and DNA transfer and repli-
cation (Dtr) system genes (trfAp and traJ), as well as the 16S rRNA
gene that was used to normalize gene expression, were evaluated
in the present study. All PCR primers are listed in Table S2, the
detailed procedures for RNA extraction and qRT-PCR are presented
in Text S3 (Zhang et al., 2017), and the technical parameters of qRT-
PCR are shown in Fig. S1.

2.8. Statistical analysis

Each experiment was conducted at least triplicate. Significant
differences were statistically estimated using analysis of variance
(ANOVA) and an independent sample t-test using SPSS 23.0 (IBM-
SPSS, Chicago, IL, USA). A value of $P < 0.05$ was noted to be sig-
ficant, and a value of $P < 0.01$ was noted to be very significant, ac-
cording to previous studies (Zhang et al., 2017).

3. Results and discussion

3.1. Effect of sub-inhibitory concentrations of heavy metals on
conjugal transfer

The exposure sub-inhibitory concentrations of each metal ion
used in this study (Table S3) were selected based on MICs deter-
mined in the present and previous studies (Nies, 1999), environ-
mental standards (GB15618–2008; GB3838–2002; USEPA, 2009;
WHO, 2011), and the detected environmental concentrations (Teng
et al., 2014; Waseem et al., 2014). As shown in Fig. 1, these metals
impacted the conjugal transfer frequency in a concentration-
dependent manner with initially enhanced transfer rates as the
metal concentrations increased from 0.005 to 0.05 mg/L Cu(II),
0.002–0.02 mg/L Ag(I), and 0.1–1.0 mg/L Cr(VI), respectively. Above
certain threshold concentration, which varies for different metals,
the impact started to decline. Based on maximum fold changes in
conjugal transfer rates, these metal ions were ranked Cu(II) > Ag(I) > Cr(VI) > Zn(II) in order of their ability to facilitate
the conjugal transfer of ARGs at sub-inhibitory.

Enhanced horizontal transfer of ARGs by V(III), Ca and Hg, be-
tween marine Photobacterium and E. coli, in a concentration-
dependent manner was reported by Suzuki et al. (2012). And
their results indicated that treatment with inhibitory levels of these
metals decreased the frequencies of conjugal transfer (Suzuki
et al., 2012). Our results, for the first time, showed that heavy
metal ions, such as Cu(II), Ag(I), Cr(VI), and Zn(II), at environ-
mentally relevant sub-inhibitory concentration ranges, may contribute
to the enhanced horizontal transfer of ARGs. This implies that the
non-biodegradability and widespread occurrences of sub-
inhibitory levels of heavy metals may present long-term environ-
mental implications in influencing the dissemination of ARGs,

3.1.2. Effect of Cu(II), Ag(I), Cr(VI), and Zn(II) on E. coli K12 costylic ROS production

Metal ions had significant effects on the ROS formation in E. coli
strains (ANOVA, $P < 0.05$); significant differences between individual metal ion treated groups and the
control (0mg/L of metal ion) were tested with independent sample t-test and shown with “* ($P < 0.05$), “** ($P < 0.01$).

Fig. 2. Relative ROS production levels in donor (E. coli S17-1) (a) and recipient
(E. coli K12) (b) bacteria upon exposure to Cu(II), Ag(I), Cr(VI), and Zn(II) at sub-
inhibitory concentrations. The error bars represent ± standard error of the mean. The ROS production (increased approximately
1–1.5 folds) significantly positively correlated to the conjugative transfer by linear
regression analysis. Fold changes in transconjugant relative to untreated controls
(no heavy metal treatment) and treatment with 100 mM TU and 0.01 mg/L Cu(II),
0.01 mg/L Ag(I), 0.1 mg/L Cr(VI), and 0.05 mg/L Zn(II) (b). Significant differences be-
tween individual metal ion treated groups and the control (0 mg/L of metal ion) were
tested with independent sample t-test and shown with “* ($P < 0.05$) and “** ($P < 0.01$).
which might have been underestimated.

3.2. Mechanisms involved in enhanced conjugative transfer by sub-inhibitory concentrations of heavy metals

Previous reports have shown that ROS formation was responsible for the promotion of horizontal transfer caused by sub-inhibitory concentrations of antibiotics and disinfection byproducts (Andersson and Hughes, 2014; Beaber et al., 2004; Baharoglu et al., 2013). Considerable evidence suggests that ROS generation plays an important role in metal-induced toxicity (Lemire et al., 2013). Therefore, we hypothesized that ROS generation, subsequent induction of oxidative stress and SOS response, increases in cell membrane permeability, and changes to conjugation-related genes caused by heavy metals, even at sub-MIC concentrations comparable to those present in the environment, may facilitate conjugative transfer.

3.2.1. Sub-inhibitory levels of heavy metals lead to intracellular ROS generation

Oxidation by ROS is responsible for damage to cellular structures and, ultimately, cell death (Lemire et al., 2013). In this study, levels of ROS production in both donor and recipient strains increased significantly with increasing concentrations of metal ions (Fig. 2). Linear regression correlation analysis between the ROS production and the changes in the conjugative frequency showed significant positive correlation ($P < 0.05$) (Fig. 3a). The addition of the ROS scavenger (thiourea) significantly reduced conjugative transfer (Fig. 3b), which evidenced that moderate ROS production induced by metals likely contributed to or associated with the promotion of conjugative transfer. Recent studies indicated that sub-inhibitory levels of antibiotic (Baharoglu et al., 2013) and disinfectants (Zhang et al., 2017) can accelerate horizontal transfer of ARGs by increasing intracellular ROS formation, and subsequent induction of multidrug efflux systems, interference on tRNA synthetase, and ROS-induced DNA mutagenesis (Zhang et al., 2017; Andersson and Hughes, 2014; Beaber et al., 2004; Baharoglu et al., 2013; Lebreton et al., 2012).

3.2.2. Transcriptional analysis of the impact of metal ions on oxidative stress and SOS response

We performed a quantitative, real-time transcriptional assessment of the representative genes in the oxidative stress and SOS response pathways that are known to be involved in antibiotic resistance (Zhang et al., 2017; Li et al., 2016). The specific and dynamic gene expression profiles were obtained for E. coli exposed to sub-inhibitory concentrations of four metals for 2 h (Fig. 4a). As expected, these sub-inhibitory levels of metal ions altered the transcriptional activity of certain genes involved in oxidative stress and SOS response pathways. Specifically, exposure to 0.05 mg/L Cu(II), 0.01 mg/L Ag(I) and 0.1 mg/L Cr(VI) led to a significant up-regulation of oxidative stress genes (soxS, soxR, oxyR, dps, katG, sodA, ahpC, and katE), as well as SOS response genes associated with DNA damage and repair (recX, ybfE, recN, sulA, uvrD, lexA, umuD),
mutT, and dinB) (Fig. 4a and Table S1). In comparison, Zn(II) induced relatively lower magnitude in altered gene expression in oxidative stress and SOS response, which may be associated with the in vivo antioxidant activity of zinc (Powell, 2000). Our results are in agreement with the metal-induced formation of free radicals and their stimulation of oxidative stress and the SOS response (Lemire et al., 2013).

Three transcription factors (TFs), OxyR, SoxR, and SoxS, play critical roles in transcriptional regulation of the defense system for oxidative stress in bacteria (Seo et al., 2015). The activation of SoxR enhances transcription of the gene soxS, and the SoxS protein increases resistance to oxidants and antibiotics (Kabir and Shimizu, 2006). The present results indicated that the soxS, soxR and oxyR genes were significantly up-regulated when exposed to sub-inhibitory levels of these four metal ions (Table S1), likely contributing to the enhanced conjugative transfer.

Previous studies indicated that the induction of the SOS response could promote horizontal transfer of ARGs because of the influence on expression of conjugation-related genes and homologous recombination (Lv et al., 2015; Martinez et al., 2006; Samuels et al., 2000). The present results also showed that these four heavy metal ions could generate intracellular ROS (Fig. 2), and consequently, induce oxidative stress and the SOS response (Fig. 4a). RecA and lexA genes are up-regulated when the SOS response is turned on (Baharoglu et al., 2013). Similarly, the mRNA expression levels of genes (recA, lexA, and umuD) involved in the SOS response were up-regulated when exposed to sub-inhibitory concentrations of metal ions, compared with controls (Fig. 4a), which likely consequently mediated the enhancement of conjugative transfer.

3.2.3. Sub-inhibitory metal ions affected cell membrane permeability

Membrane permeability is associated with the structural components of bacterial membranes, which form a selective hydrophobic lipid bilayer barrier embedded with specific pore-forming proteins (Delcour, 2009). Our FCM results showed that Cu(II), Ag(I), Cr(VI), and Zn(II) caused damage to membrane integrity (Fig. 5 and Fig. S3). Particularly, 0.01 mg/L Ag(I) and 0.01 mg/L Cu(II) increased the percentage of membrane-damaged *E. coli* S17-1 and *E. coli* K12 cells by 1.32- and 1.33-fold, respectively, compared with controls (Fig. S3).

Our real-time transcriptional analysis data showed that
expression of the lipid-associated genes rfaZ, rfaQ, pmrB, htrI, and pslX was impacted by heavy metals at sub-inhibitory concentrations (Fig. 4b and Table S1). In previous research, the results of thiobarbituric acid-reactive substance (TBARS) assays indicated that metal exposure could lead to impaired membrane function through lipid peroxidation (Anand and Kanwar, 2001). Cu(II) is a redox-active metal that can induce ROS production, while Cd(II) as a non-redox-active metal that can deplete free-radical scavengers, and both metals are, therefore, capable of causing oxidative damage to cellular membrane phospholipids and the loss of membrane impermeability, albeit through different mechanisms (Lemire et al., 2013).

The outer membrane (OM) is a selective barrier that contains specialized protein channels and receptors for specific substrate binding and uptake (Delcour, 2009). We investigated the expression of two typical OM protein genes, OmpA (34 kDa) and OmpC (36 kDa), by qRT-PCR. The results demonstrated that certain sub-inhibitory concentrations of Cu(II), Ag(I), Cr(VI), and Zn(II), which enhanced conjugative transfer as described above, significantly up-regulated the expression of ompA and ompC (Fig. 6). Specifically, 0.01 mg/L Cu(II), 0.01 mg/L Ag(I), 0.1 mg/L Cr(VI) and 0.05 mg/L Zn(II) increased the expression of ompA by 5.88-, 4.40-, 2.14- and 3.68-fold, and up-regulated ompC by 3.11-, 3.48-, 2.78- and 7.24-fold, respectively. Importantly, the expression of ompA and ompC increased with increasing heavy metal ion concentration within certain concentration range, then the expression was down-regulated at higher concentrations above a metal-specific threshold (Fig. 6). This may be because metal ions bind to outer-membrane proteins, which may decrease their uptake into cells (Mermod et al., 2012). Previous investigators proposed that more OmpA and OmpC proteins contributed to increased cell adhesion between receptors, which could enhance the conjugation efficiency via direct cell-to-cell contact (Schröder and Moser, 1997).

3.2.4. Effects of sub-inhibitory concentrations of metal ions on the expression of conjugative transfer-related genes

Global regulatory genes (korA, korB, and trbA) are known to be responsible for regulating the transfer, replication, and stable maintenance of plasmid pCM184-Cm during conjugation (Samuels et al., 2000). As shown in Fig. 7a and Fig. S4, mRNA levels of korA, korB, and trbA were down-regulated to varying degrees following exposure to 0.01 mg/L Cu(II), 0.01 mg/L Ag(I), 0.1 mg/L Cr(VI), and 0.05 mg/L Zn(II), respectively, compared with controls. Specifically, korA expression was reduced by 56.8%, 33.2%, 10.1% and 44.3%, respectively. Cu(II) induced a higher conjugative transfer than the other metal ions tested (Fig. 7a and Fig. S4).

Down-regulation of global regulatory genes can activate the mating pair formation (Mpf) system, which is involved in forming conjugative bridges between cell surface proteins during the early stages of conjugation (Zatyka et al., 2001). Indeed, repression of korB and trbA significantly up-regulates the expression of trbB gene promoters (Zatyka et al., 2001). Particularly, exposure to 0.01 mg/L Cu(II), 0.01 mg/L Ag(I), 0.1 mg/L Cr(VI), and 0.05 mg/L Zn(II) resulted in the up-regulation of trbBp in the present study by 2.1-, 5.1-, 4.4- and 3.6-fold, respectively (Fig. 7b). Induction of trbB expression is essential for facilitating the assembly of cell surface or membrane-associated channels, including the pilus needed for the transmission of single-stranded DNA (Zatyka et al., 2001). Another Mpf gene, traf, was also up-regulated approximately 5-fold in response to sub-inhibitory metal ions, compared with controls (Fig. 7b).

Global regulatory genes are also associated with the genes involved in DNA transfer and replication (Dtr) systems, such as rfaA and traf. For example, korA and korB can potentially repress expression from the rfaA promoter (rfaAp), thereby decreasing the single-stranded and DNA-binding protein TrfA that contributes to activation of the replication origin (Kostelidou et al., 1999). As illustrated in Fig. 7b, sub-inhibitory Cu(II), Ag(I), Cr(VI), and Zn(II) significantly up-regulated trfA expression in E. coli S17-1 and E. coli K12 by 1.9–2.7-fold, compared with controls. Furthermore, traf, another Dtr system gene that preforms a crucial role in relaxosome formation, was also up-regulated following metal treatments (Fig. 7b and Fig. S4). This is relevant because the relaxosome introduces a site- and strand-specific nick at the origin of transfer (oriT), from which conjugal DNA transfer is initiated (Furuya and Komano, 2000).

4. Conclusion

This study demonstrated that heavy metals, including Cu(II), Ag(I), Cr(VI), and Zn(II), can accelerate horizontal transfer of ARGs when present at sub-inhibitory concentrations comparable to those found in polluted environments and in treated animals and humans, thereby possibly enhancing the development and transmission of antibiotic-resistant and potential pathogens. This study provides evidence from multiple aspects that approve the ecological effect of low levels of heavy metals on antibiotic resistance dissemination and implies the urgency of strengthening efficacious policy and technology to control heavy metal pollutants in various environments.

The underlying mechanisms for enhanced conjugative transfer...
facilitated by sub-inhibitory level of various metal ions have been systematically explored by using physiological, biochemical, and genomic approaches and are revealed to involve intracellular ROS generation, increasing cell membrane permeability, the induction of oxidative stress and the SOS response, and altered expression of genes involved in conjugative transfer. This is consistent with the previously recognized analogies between the mechanisms by which sub-inhibitory antibiotics influence conjugative transfer (Baharoglu et al., 2013) and metal toxicity mechanisms implicating metal resistance systems (Asakura et al., 2009). Metal-induced toxicity involves compromising the structural and functional integrity of cell membranes, inducing oxidative stress, and inflicting chromosomal damage (Lemire et al., 2013; Asakura et al., 2009). Even at extremely low concentrations, certain heavy metals can cause genetic toxicity in microbes (Lemire et al., 2013; Asakura et al., 2009). Based on these findings, we hypothesize that low levels of other toxic heavy metals, such as mercury, arsenic and nickel, may also have the potential to promote the transfer of ARGs through conjugative mechanisms. In addition, further research on the mixed effects of heavy metals is much needed, to elucidate the potential contribution of combined heavy metals, as often occur in the polluted environment, to antibiotic resistance phenomena via mechanism involving enhanced horizontal transfer of ARGs.

Summary of the main findings

This study revealed that sub-inhibitory heavy metals promoted conjugative transfer of ARGs, and mechanisms involved ROS formation, SOS response, membrane permeability and altered gene expression.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.01.032.

References


United States Environmental Protection Agency (USEPA), 2009. National Primary and Secondary Drinking Water Regulations. EPA, 816-F-09-004.


