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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 theminimum 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.

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

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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 frequencies of conjugative transfer (Suzuki et al., 2012; Martinez et al., 2006). However, metals (e.g., Cu, Zn, Cd, Cr, Pb, 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 difference 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 $CuSO_4 \cdot 5H_2O$, Ag_2SO_4, $K_2Cr_2O_7$ and $ZnSO_4 \cdot 7H_2O$, 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,