Acceleration of emergence of E. coli antibiotic resistance in a simulated sublethal concentration of copper and tetracycline co-contaminated environment

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Abstract

A co-contaminated environment with metals and antibiotics ultimately provides exposing of bacteria to metals and antibiotics at the same time and prevails co-contamination in the environment. This study objective, to explore the efficacy of sublethal concentrations of copper ions contaminated with tetracycline to antibiotic resistance in sensitive strain *E. coli* K12. We proved that copper ions and tetracycline co-contaminated environments could considerably enhance the mutation frequencies of chloramphenicol and polymyxin B resistance in antibiotic susceptible *E. coli*; however, the corresponding copper ions and tetracycline alone showed weaker effects. Results also demonstrated that the relatively high sublethal concentrations of copper ion and tetracycline co-contaminated environment could induce much higher antibiotic resistance compared to the low sublethal and the control groups. Whole-genome characterization results indicated the variability of genotype and phenotype involved in antibiotic resistance. In addition, the evolved resistant strains displayed hereditary resistance after 5 round culture cycles in LB broth over 5 days. Results implied that co-contamination with metals and antibiotics environment could fortify the resistance, and might further contribute to the induction and dissemination of antibiotic resistance in metal and antibiotic co-contaminated environment.

Key Points

- *Mutation frequencies increased with cross contamination.*
- *Antibiotic resistance induction was dose dependent.*
- *Genotype and phenotype could diverge under the same stress condition.*
- *The resistant mutants displayed hereditary resistance.*

Introduction

Antibiotics are the essential double-edged sword: one hand, antibiotics are a powerful weapon to life-saving, on the other hand, misuse and overuse urge bacteria development and dissemination of resistance (Zhu et al., 2013; Lv et al., 2014; Vikesland et al., 2017). Antibiotic resistance forms an increasing risk to our body health, since this is relevant to the losing of antibiotic therapy ability (Vikesland et al., 2017). The rise of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) determined in clinical as well as nature, because the over-use of antibiotics induced the selective stress in our environment (Alonso et al., 2001). Residual antibiotics from human and animal feces/wastes, hospital waste and pharmaceutical industries eventually contaminate the soil and water environments (Pruden, 2006; Nolivos et al., 2019; Povolo and Ackermann, 2019). In addition, soil fertilizer with animal waste and sewage sludge are causing antibiotic accumulation (Zhu et al., 2013). Also, residual antibiotics could run away from the soil environment, then get to the water ecosystem (Martinez, 2008). Furthermore, antibiotics are regarded as “pseudo-persistent”, although the half-life of many antibiotics is relatively short, their continued presence in the environment (Pan et al., 2011).
Moreover, heavy metals are persistent in nature, gathering in a diverse constituent of the ecosystems (Lu et al., 2014). Copper and zinc are generally employed as food additive in animal feed diets through the supplement of the ingredients in their compound feed, exceeding the needs for normal growth of the animals, and for precaution of animal disease, as well as for the growth promotion and medical remedies (Baker-Austin et al., 2006; Hau et al., 2017). Reports claimed that copper in the soil, not only selects for copper resistance but also co-selects for resistance to antibiotics, for example, chloramphenicol, tetracycline and ampicillin (Berg et al., 2005). In addition, more research related to antibiotic resistance is focused on high concentrations, such as, more than the minimal inhibitory concentration (MIC) (Seiler and Berendonk, 2012), while the effect of relatively low concentrations, that is sublethal (< MIC) still mainly unknown.

Interestingly, several reports displayed that far lower than the MIC concentration of the metal can endow bacteria antibiotic resistance through co-selection, as well as heavy metal resistance (Gullberg et al., 2014; Chen et al., 2015). Importantly, a considerable number of research indicated that heavy metal pollution in environments could contribute to the maintenance and dissemination of antibiotic resistance (Stepanauskas et al., 2005; Henriques et al., 2016; Zhang et al., 2018b; Imran et al., 2019). Such as, Zhu et al. (2013) reported a significant correlation between copper concentration and the incidence of ARGs (Zhu et al., 2013). In some natural ecosystems, previous studies have shown that heavy metals and antibiotics co-contaminated together could drive the dissemination of antibiotic resistance in bacteria (Baker-Austin et al., 2006; Wang et al., 2014), and co-exposure to zinc and antibiotic co-contaminated environments such as oxytetracycline in activated sludge bioreactors could enhance the resistance of the microbial community towards antibiotics (Peltier et al., 2010).

In this study, we simulated copper ions and tetracycline co-contaminated environment through evolutionary experiments to determine whether sublethal of copper ion contaminated with tetracycline could increase the bacterial antibiotic resistance. Besides, whole-genome sequencing analysis was conducted to insight into the potential mechanisms. Moreover, comparative molecular analysis of resistance determinants, coupled with phenotypic analysis, may contribute to further understanding of sources of antibiotic resistance. The findings of this study will make clear on the complex relationship between heavy metals and antibiotics co-contamination and antibiotic resistance. And, further studies on the changes in resistance with generations on the evolution cycle in E. coli will be summarized in our next study.

**Materials And Methods**

**Strains, antibiotics and selection condition**

First, E. coli K12 (MG1655) was been sequenced (Shanghai Majorbio Bio-pharm Technology Co., Ltd), which was designated as the original wild type strain (Table 1). Activating E. coli from storage tube with glycerol stock which stored in -80 °C, expanding propagating on a Luria Bertani (LB) agar plates, cultured
at 37 °C for 16 h. The selected seed strain was cultured in LB broth at 37 °C for 12 h for following selected experiments (Li et al., 2016).

The involved antibiotics: chloramphenicol (Chl), ciprofloxacin (Cip), erythromycin (Ery), gentamycin (Gen), tetracycline (Tet), and polymyxin B (Pol) and cupric (CuSO4·5H2O) were get from Solarbio, Inc. (Shanghai, China). 90 % inhibition of growth was regarded as the MIC of each antibiotic, which was determined as Test S1 described, and the MIC of copper ions was also investigated with the same method. The detailed accounts are displayed in Text S1. The tetracycline resistant cultures were kept from light so as not to degrade the antibiotic.

**Determination of minimum inhibitory concentrations**

The MICs were investigated after 40 sub-culture cycles, using previously described methods (Lv et al., 2014). The serially diluted strain cultures were grown on LB agar plates for 16h at 37 °C. Then 10 colonies from each parallel agar plate were unintentionally selected from each sub-culture. These randomly picked strains were cultured in 3 mL of LB broth for 5-6 h at 37 °C to investigate the MICs (Text S1) (Li et al., 2016). And, the MICs of the original *E. coli* K12 strain was also determined (Table 1).

**Exposure to copper ions and/or tetracycline environment**

Detailed procedures of the exposure experiments are shown in Fig 1. First, a total of 5 mL strains, including 0.5 mL of original *E. coli* cultures (10^6 CFU) and 4.5 mL of fresh LB broth which contained corresponding concentrations of stated copper ions and/or tetracycline, cultured in a 15 mL sterilized tube at 37 °C for 24 h. Second, 0.5 mL of the cultures was transferred into a new 15 mL sterilized tube with 4.5 mL fresh LB broth to sub-culture with corresponding copper ions and/or tetracycline. The original isogenic K 12 cultured in LB broth lacking any copper ions or tetracycline as the control. All performance in triplicate.

The exposure dosages of copper ions and tetracycline applied in this research, according to the MICs as well as the concentration of environments (Fig. S1, Table 1), in view of the tested and evaluated environmental concentrations (Zhu et al., 2013), such as the cupric concentration in Dawu River ranged from 12 to 30 mg/L (Huang Changgan 2004), and the cupric concentration previous studies ranged from 8 to 500mg/L (Chen et al., 2015; Poole, 2017). The resistance evolution experiments were observed in five conditions (Fig. 1b), gradually increasing dose concentrations from 1/100×MIC (20 mg/L) up to 1/10×MIC (100 mg/L) (as the relatively high concentration evolution condition) and relatively lower concentrations of copper ions about 10 mg/L, which were the main two differences. In the relatively high concentration evolution selection culture, the concentrations of copper ions gradually raised from an original of 20 mg/L to eventual about concentrations of 100 mg/L (recorded as H), respectively (Fig. 1a). In the relatively low concentration evolution cultures, the concentrations of copper ions and tetracycline were kept consistent pattern at 10 mg/L and 0.0234 mg/L (recorded as L). All experiment manipulations were manipulated in triplicate.
We mentioned the initial bacterial culture with copper ions and/or tetracycline treatments or without any copper ions and/or tetracycline (control groups) recorded as C0. The following sub-cultures were devised as cycle 1, cycle 2, etc., recorded as C1, C2, etc., respectively (Fig. 1a). 4 mL of the sub-culture of every experimental cycle was mixed with glycerol solution in a 15% (v/v), then stored in -80 °C for next step (Li et al., 2019). All culture steps were incubated with 200 rpm shaking in aerated incubators, and each cycle was 24h, a total of 40 sub-culture cycles (Fig. 1a).

**Subsequent antibiotic resistance determination**

After 40 cycles of sub-culture, the mutation rates were investigated (Kohanski et al., 2010). To determine the antibiotic resistant mutation rates, sub-cultured strains were streaked on LB agar plates containing corresponding antibiotics (Table 1), following cultured for 48 h at 37 °C (Li et al., 2016), and the colonies were counted. The colonies grew on LB agar without any antibiotics were regarded as the total bacterial concentrations. Whereas, the colonies grew on LB agar plates containing antibiotic were also determined as resistance to the corresponding antibiotics (Lv et al., 2014). The maximum-likelihood methods was used to investigate the mutation frequency as previous described (Lv et al., 2014), as the follow formula (a).

\[
\text{Mutation rates} = \frac{\text{Resistant clones}}{\text{Total number of clones}} \quad (a)
\]

Fold changes of mutation rate were measured through each exposure treatment relative to an untreated control group.

**Hereditary stability determination**

Hereditary stability of the randomly selected clones after 40 cycles was tested for ve days of sub-culture cycles (Lv et al., 2014; Zhang et al., 2018c). That is, the selected strains were diluted 1:100 in 5mL of fresh LB media without any treatment, then regrowth for 24h at 37 °C, 180 rpm. Each selected strain was exposed to 5 such cycles of growth (Zhang et al., 2018c). The MIC values were measured after 5 days of sub-culture, then matched the MICs of the initial strains to determine the hereditary stability. The MIC determination method was used as former research described (Li et al., 2016; Khan et al., 2017) (Text S1).

**DNA extraction and whole-genome sequencing**

The strains from each treated group and the control group cultured 2 to 3 times on LB agar plates without any antibiotics for 16 h at 37 °C. The Universal Genomic DNA Extraction Kit (Takara, Beijing, China) was used to extract the total DNA, the manipulation process was depending on the manufacturer’s instructions. The concentration and purity were investigated by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE).
The NEXTflex™ Rapid DNA-Seq Kit was applied to set up Illumina sequencing libraries. Briefly speaking, 5’ prime ends were first end-repaired and phosphorylated. Next, the 3’ ends were A-tailed and ligated to sequencing adapters. The third step was to enrich the adapters-ligated products using PCR (Shanghai Majorbio Bio-pharm Technology Co., Ltd).

**Data analysis**

Microsoft Excel 2016 (Microsoft Inc., USA) was used to manipulate the mean, standard deviation and fold change of data. Independent sample t-test was used to analysis significant differences (SPSS 18.0, USA).

**Results**

**Copper ion and tetracycline co-contaminations enhance the mutation frequency of polymyxin B and chloramphenicol**

Figure 2 indicates the effects on bacterial antibiotic resistance after exposing the *E. coli* strains to the pressure condition of sublethal concentrations of copper ion and/or tetracycline. The mutations rate of the control which was not treated by sublethal concentrations of copper ion and/or tetracycline was regarded as the spontaneous mutations rate, which ranged from $10^{-8}$ to $10^{-6}$ for different antibiotics.

Sublethal concentrations of copper ions and tetracycline co-contaminated treatment could critically enhance the resistant mutation rates of polymyxin B and chloramphenicol ($P < 0.05$) (Fig 2a/b), compared with the control groups. The increased of the mutations rate of resistance to ciprofloxacin and gentamicin were < 2fold compared to the control groups (data is not displayed). Sublethal concentrations of copper ions and tetracycline co-contamination resulted in copper dose-dependent alteration in resistance mutation rate for polymyxin B (Fig 2a) and chloramphenicol (Fig 2b), and the higher of the copper concentration of the compounds the higher of polymyxin B or chloramphenicol resistance, copper ions treatment alone was the same.

The resistance of *E. coli* K12 to polymyxin B or chloramphenicol showed that relatively high sublethal concentrations of copper ion acting alone or in combination with antibiotics can increase the frequency of mutations, such as 0.2-2.9, 18.8-41.9, 3.1-6.1 and 32.9-36.8 folds respectively, compared with the controls. However, only treatment with relatively low sublethal of copper ion and tetracycline co-contamination caused greatly increase in the mutation rates for tetracycline resistance, and the increase up to 1730.8 folds, but only 5 folds increase with relatively high sublethal of copper ion and tetracycline co-contamination (Fig 2c). In addition, both low and high sublethal of copper ion treatment induced mutant resistance to tetracycline with a 0.04- and 0.44- fold increase, respectively (Fig 2c), and resistance to tetracycline up to 3.64 folds with tetracycline treatment (Fig 2d).

**Effects of sublethal concentration of copper ion and tetracycline co-contaminations on antibiotic resistance**
The untreated control samples grown on LB broth were not determined against the antibiotics competed with the original *E. coli* K12 strains (Fig 3a). With a sublethal concentration of copper ion and/or tetracycline exposure, selected strains that demonstrated clinically relevant resistance to ciprofloxacin, erythromycin, polymyxin B, tetracycline and chloramphenicol with an increase in MICs by 1 to 32 folds (Fig 3a). The resistance to gentamycin was no more than 1-fold raise in MICs (Fig 3a). The average MICs for sublethal copper ion and tetracycline co-contaminated environment selected ciprofloxacin-resistant strains were more than 11-16 and 16-20 folds higher than that of the treatment with copper ion alone and the original or LB-control *E. coli*, respectively (Fig 3b). For ciprofloxacin, the relatively high sublethal of copper ion contaminated with tetracycline antibiotic-selected resistant strains displayed over 4-folds higher MICs than the relatively low sublethal, and tetracycline-selected ciprofloxacin resistant strains exhibited 5-folds higher than the original or LB-control *E. coli* (Fig 3b). The tetracycline and chloramphenicol resistant strains selected with relatively low sublethal of copper ion treatment showed higher resistance compared with baring to copper ion and tetracycline co-contaminated environment (Fig 3c/d).

**Whole-genome sequence analysis of the evolution strains**

To determine the underlying genetic mechanisms involved in antibiotic resistance caused by sublethal of copper ions and/or tetracycline environments, whole-genome sequencing of the selected mutants was operated. 2-3 isogenic clones were selected from the strains tackled by relatively low or high sublethal concentrations of copper ions and/or tetracycline to whole genome sequencing analysis (Brockhurst et al., 2011).

Three genetic changes involved in ARG were determined, that is *mdtF*, *macB* and *vanRI*, including a substitution mutation and insertion genetic changes induced by copper ion and/or tetracycline exposure (Fig. 4, Tables 2). Two different changes were respectively identified on *mdtF* and *vanRI* genes in the LB control strain, maybe caused by spontaneous mutations throughout the sub-culture processes, such as caused by growing situation and likely stress (Table 2). One genetic mutation in 1 gene was detected in *E. coli* strains induced by a relatively high sublethal copper ions environment (Fig. 4 and Tables 2). The genetic insertion was associated with membrane transporter gene (*mdtF*), for the mdtEF-TolC efflux complex. Similarly, one genetic mutation in 1 gene was detected in *E. coli* strains caused by relatively low sublethal copper ions or tetracycline treatment and relatively low sublethal of their co-contaminated environments, which was associated with transcriptional activator in the vanSR regulator within the vanI glycopeptide resistance gene cluster (*vanRI*) (Fig. 4 and Tables 2). The number and evolution of clinically relevant antibiotic resistance genes could be enhanced by contaminations, while possibly as well as many other genes with extraordinary main functions but with a resistance phenotype present in the environmental resistome.

**The hereditary stability of antibiotic resistance in evolved *E. coli* strains**

Hereditary antibiotic resistance in the evolved strains exposure to sublethal concentration of metal and antibiotic co-contaminations is great significance for human health and environmental safety (Lv et al.,
Fig 5 showed the MICs of ciprofloxacin-, tetracycline- and chloramphenicol-resistant after 5 round culture cycles in LB broth over 5 days. Results indicate that the resistance levels to tetracycline and chloramphenicol significantly decreased by relatively low sublethal of copper ion or the relatively high sublethal of the co-contaminations treated (Fig 5b, 5c), and conversely, the resistance levels to ciprofloxacin increased significantly with copper ion and/or tetracycline treatment except for the relatively low sublethal of copper ion (Fig 5a).

**Discussions**

The present data analysis showed that sublethal of copper ions and tetracycline co-contamination could enrich *de novo* resistant mutants (Kohanski et al., 2010), to increase the mutation frequencies of the corresponding antibiotics. Furthermore, a previous study indicated that environmental pollutions such as metals, biocides and organometallics can promote the transmission of mobile genetic elements (MGE) through co-selection mechanism, thereby contributing to antibiotic resistance. Research reports also proposed that extremely low concentrations of antibiotics (Kohanski et al., 2010; Gullberg E and DI, 2011), heavy metals (Li et al., 2019), disinfectants (Kohanski et al., 2010), and disinfection by-products (DBPs) (Lv et al., 2014; Li et al., 2016) could induce bacteria evolving antibiotic resistant. Hence, along with previous reports (Gullberg et al., 2014; Chen et al., 2015), we hypothesized that the presence of ARB in varied sublethal concentrations of copper ions and tetracycline environments are partially understood through the selective effects of co-contaminated environmental chemicals, and the co-selection of co-contaminations driven *E. coli* evolved antibiotic resistance via co-resistance or cross resistance.

In addition, many studies proposed that, environmental pollutions, such as metal, can accelerate the dissemination of ARGs (Kohanski et al., 2010; Zhang et al., 2018b). This study suggests that heavy metals and residual antibiotics may combine to long-term stress on antibiotic resistance populations.

The MICs determination was performed, in order to investigate phenotypic evidence of resistance to antibiotics (Toprak et al., 2011), and the MICs were investigated to evaluate the bacteria resistance (Li et al., 2016). In this study, *E. coli* displayed the highest resistance to sublethal concentrations of copper ion and tetracycline co-contaminated treatment, while the relatively high sublethal of the co-contamination were the most toxic compared with the relatively low sublethal or their action alone (Fig. 3). It is becoming increasingly clear that the environment in which organisms exist affects their susceptibility to antibiotics. This is confirmed by the selection and enrichment of antibiotic-resistant organisms without antibiotic exposure (Kohanski et al., 2010). All common sources of environmental stress are metal ions, especially copper (Cu) and zinc (Zn), which are essential for the function of normal bacterial cells at low concentrations, but are essential for some metalloproteins (Foster et al., 2014), and toxic at a high level (Lemire et al., 2013). Eventually, at relatively higher levels, these metals provide selective stress for metal resistance, which in turn is driven by genetic and physiological links between the two resistances (Fig. 4). In environments contaminated with copper and tetracycline, the simplest reason for the enrichment of antibiotic-resistant organisms may be the selection of organisms on chromosomes or plasmids that carry resistance genes for both drugs (Table 2, Fig. 4).
Previous reports suggested that chromosomal mutation is the primary way and mechanism for bacteria to acquire ARGs, such as base substitution or frameshift in specific genes (Kohanski et al., 2010). Several research reports have proclaimed that sublethal concentrations (far below the MIC) of antibiotics also can induce results in mutation rates increased for bacteria. Furthermore, these mutations appear at a high frequency due to the relatively low fitness-cost (Gullberg E and DI, 2011). This phenomenon indicated that sublethal of antibiotics are good at the acquiring of ARGs (Kohanski et al., 2010; Gullberg E and DI, 2011). Hence, this may explain with sublethal concentration of copper ion and tetracycline exposure, selected strains that demonstrated clinically relevant resistance to ciprofloxacin, erythromycin, polymyxin B, tetracycline and chloramphenicol with a significant increase in MICs.

The mdtF is a multi-antibiotic inner membrane transporter for the mdtEF-TolC efflux complex. It is a resistance nodulation division (RND) type efflux pump in E. coli, with important homology to acrB, but it generally expressed at a low level in clinical isolates. A previous study indicated that overexpressed pumps can induce susceptibility to erythromycin (Bohnert et al., 2007). In fact, all Gram-negative strains have genes for efflux pumps that are the part of the RND family, and these pumps are quite effective in developing resistance to multiform complex mixtures, for example, antibiotics, dyes and other xenobiotics, even multidrug resistance (MDR) (Jellen-Ritter and Kern, 2001; Webber et al., 2005). So, the various MICs of most of the antibiotics were involved in the detection of single point mutation. This single-point mutation increases the resistance to exposed antibiotics but reduces the resistance to another antibiotic (Bohnert et al., 2007). From this point of view, we able to explain why the phenotype and genotype are inconsistent in this study. In addition, macA is involved in the membrane fusion protein (MFP), while macB is an ATP-binding cassette (ABC) transporter, which shaping an antibiotic efflux mix with macA and TolC (Kobayashi et al., 2003; Elena B. Tikhonova, 2007), which was the first ABC-type drug exporter experimentally identified in a Gram-negative bacterium (Kobayashi et al., 2003; Elena B. Tikhonova, 2007). Thus, an important feature involved in antibiotic resistance of macB is the cooperation with TolC for antibiotic efflux (Kobayashi et al., 2001). Previous studies have shown that the gonococcal macA–macB efflux pump could identify macrolides, and its action with the mtrC–mtrD–mtrE pump could reduce the susceptibility of gonococci to macrolides (Rouquette-Loughlin et al., 2005). Although the macA–macB efflux pump could enhance the strains resistance to macrolides when overexpressed in an E. coli background, however, only marginally reduced antibiotic resistance to azithromycin and erythromycin (Rouquette-Loughlin et al., 2005). Finally, vanRI is a regulatory activator of transcription in vanSR regulators within the vanI glycopeptide resistance gene cluster (ARO:3003728), glycopeptide resistance gene which conferring antibiotic resistance via molecular bypass. Thus, the mdtF, macB and vanRI mutation might contribute to developing ciprofloxacin, chloramphenicol and polymyxin B resistance in E. coli (Fig. 2–3). The above finding indicates that at the relatively high copper ion sublethal (below 1/10 of the MIC) contaminated with tetracycline in polluted environments could contribute to the increase of mutation frequency. In this study, the number and uniqueness of genetic alteration were different from mutants caused by varied sublethal of copper ions and/or tetracycline co-contaminated environments, and, phenotypes and genotypes also showed significant inconsistency.
The evolution and dissemination of antibiotic resistance are accelerated through the acquiring of ARGs via *de novo* mutation (Lv et al., 2014; Li et al., 2019). Genetic mutations are regarded as significant pathways contributes to the evolution of antibiotic resistance (Nolivos et al., 2019). In truth, the attenuation, persistence and enrichment of ARGs or ARB in the environment are ecological evolution processes that are stimulated by diverse circumstances (Andersson and Hughes, 2011).

The results of the hereditary stability of antibiotic resistance in evolved *E. coli* strains may explain by the contribution of genotypic-phenotypic discrepancies (Beaber et al., 2004; Corona and Martinez, 2013). Because strains subjected to antibiotics, no matter raised or reduced resistance level, which could relate to phenotypic changes (not-inheritable resistance) and genetic changes (inheritable resistance) (Corona and Martinez, 2013; Zhang et al., 2018a). Even so, evolved strains continued a greatly raised ciprofloxacin resistance matched the initial *E. coli* K12 and the control strains; this may obtain via genetic changes (based on the whole-genome sequencing analysis). In terms of the persistent staying alive and adaptive evolution of resistant strains, the stable hereditary features of co-contaminations-induced ciprofloxacin resistance could pose potential public health (Seiler and Berendonk, 2012; Yazdankhah et al., 2014). Thus, in this study, ciprofloxacin resistance can be stably passed on to offspring by evolved strains, this hereditary stability may cause continued to development of antibiotic resistance.

This study demonstrates a synergistic effect between sublethal doses of antibiotic and ionic copper, both individually and in combination, on stable mutations that lead to evolution of antibiotic resistant bacteria. Evidence was provided that sublethal concentrations of copper ions and tetracycline co-contaminated treatment could lead to significant increase in the evolution of polymyxin B and chloramphenicol resistant mutations. With sublethal concentration of copper ions and/or tetracycline exposure, the MICs of selected strains resistance to ciprofloxacin, erythromycin, polymyxin B, tetracycline and chloramphenicol increase from 1 to 32 folds. In addition, three genetic changes involved in the antibiotic resistance gene were determined, that is *mdtF*, *macB* and *vanRI*. Hereditary antibiotic resistance of the evolved strains showed that the evolved resistance can be lost over subsequent generations, though not always. These results might contribute to understand the emergence and dissemination of antibiotic resistance in our environment.

**Declarations**

**Declarations** Not applicable.

**Author contributions** J. L. and Z. Y. conceived and designed research. J. L. conducted experiments. I. P contributed new reagents or analytical tools. J. L., Z. Y., I. P. analyzed data. J. L. wrote the manuscript. All authors read and approved the manuscript.

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**Tables**

**Table 1. The MIC of each antibiotic to wild-type *E. coli* K12**

| No. | Antibiotics   | Abbreviation | Classification  | Stock Solution (mg/L) | MIC (mg/L) |
|-----|---------------|--------------|-----------------|-----------------------|------------|
| 1   | Ciprofloxacin | Cip          | Quinolones      | 200                   | 0.2        |
| 2   | Tetracycline  | Tet          | Tetracyclines   | 10                    | 2.34       |
| 3   | Gentamicin    | Gen          | Aminoglycosides | 10                    | 8.75       |
| 4   | Polymyxin B   | Pol          | Polypeptides    | 10                    | 0.94       |
| 5   | Erythromycin  | Ery          | Macrolides      | 6.4                   | 15         |
| 6   | Chloramphenicol| Chl          | Chloramphenicols| 30                    | 4.69       |

**Table 2. Summary of identified genetic changes in untreated control clones selected in LB medium and in strains selected in exposure environments.**
| Strains names  | Site         | Types          | Gene |
|---------------|--------------|----------------|------|
| Original *E. coli* | no genetic change |                |      |
| LB-control    | 1210-1215    | TTANGG         | mdtF |
| LB-control    | 572          | G→A            | vanRI|
| Cu-H          | 1203-1217    | GATAGGTTANTGTC  | mdtF |
| Cu-L          | 572          | G→A            | vanRI|
| Tet           | 572          | G→A            | vanRI|
| Tet+(Cu-L)    | 572          | G→A            | vanRI|
| Tet+(Cu-H)    | 572          | G→A            | vanRI|

The *mdtF* is the multidrug inner membrane transporter for the mdtEF-TolC efflux complex; *vanRI* is the regulatory transcriptional activator in the vanSR regulator within the vanI glycopeptide resistance gene cluster. H: resistant strains selected at high copper ion exposure concentrations that gradually increased up to 1/10 MIC level; L: resistant strains selected at low mental ion exposure concentrations about 1/100 MIC.