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Title:
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Date:
2018-10-01

Citation:
Kang, W., Zhang, Y. J., Shi, X., He, J. Z. & Hu, H. W. (2018). Short-term copper exposure as a selection pressure for antibiotic resistance and metal resistance in an agricultural soil. Environmental Science and Pollution Research, 25 (29), pp.29314-29324. https://doi.org/10.1007/s11356-018-2978-y.

Persistent Link:
https://hdl.handle.net/11343/282715

Title page

Short-term copper exposure as a selection pressure for antibiotic resistance and metal resistance in an agricultural soil

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Abstract:

Owing to the similar mechanisms of antibiotic and metal resistance, there is a growing concern that metal contamination may select for antibiotic resistance genes (ARGs) in the environment. Here, we constructed short-term laboratory microcosms to investigate the dynamics of a wide range of ARGs and two copper (Cu) resistance genes in an agricultural soil amended with a gradient of Cu concentrations (0~1000 mg kg^{-1}). Mobile genetic elements (MGEs) were also quantified as a proxy for the horizontal gene transfer potential of ARGs. We detected 126 unique ARGs across all the soil samples using the high-capacity quantitative PCR array, and multidrug and β-lactam resistance were the most abundant ARG categories. The copper amendments significantly enhanced the absolute and relative abundances of ARGs and MGEs, which gradually increased along the gradient of increasing Cu concentrations. The two Cu resistance genes (*copA* and *pcoR*) were highly enriched in low-level Cu treatment (50 and 100 mg kg^{-1}), and their abundances decreased with the increasing Cu concentrations. The level of metal and antibiotic resistance gradually declined over time in all Cu-amended treatments but was still considerably higher in contaminated soils than untreated soils after 56 days' incubation. Significant associations among ARGs and MGEs were revealed by the network analysis, suggesting the mobility potential of antibiotic resistance in Cu-amended soils. No significant positive correlations were found between ARGs and copper resistance genes, suggesting that these genes are not located in the same bacterial hosts. Taken together, our results provide empirical evidence that short-term copper stress can cause evolution of high-level antibiotic and metal resistance, and significantly change the diversity, abundance and horizontal transfer potential of soil ARGs.

Keywords: Copper; antibiotic resistance genes; copper resistance genes; co-selection; agriculture soil; public health

Introduction

The use of synthetic antibiotics for infection treatments and growth promotion has selected for the massive evolution of antibiotic resistance in both clinical and environmental settings (Pruden et al. 2006). The increasing prevalence and propagation of antibiotic

resistance genes (ARGs) in bacteria and pathogens have been considered as a global threat to public health and food security (Berendonk et al. 2015). The persistence of ARGs in the environment provides a likelihood of them being horizontally transferred from the environmental to human-related pathogens and other bacteria mediated by the mobile genetic elements (MGEs) such as transposons, integrons and plasmids (Rizzo et al. 2013; Sentchilo et al. 2013; Gillings et al. 2015); or being migrated into the food chain through growing vegetables in ARGs-enriched soils (Chen et al. 2017). Previous studies have reported the sequence similarity between some ARGs detected in the environment and those detected in human feces (Nesme et al. 2014) and human pathogens (Forsberg et al. 2012), indicating the potential of environmental bacteria to share their ARGs with human-related pathogens or commensal bacteria. Therefore, it is important to understand the evolution of antibiotic resistance and routes of resistance dissemination in the environment (Pruden et al. 2006), before we can include soil resistomes in appropriate mitigation options and risk assessment strategies (Ashbolt et al. 2013).

To combat the dissemination of antibiotic resistance, it is imperative to accurately assess the risks associated with environmental sources of ARGs and identify the major pathways by which resistance develops and evolves in natural settings (Hu et al. 2018). Land application of organic wastes containing a high proportion of antibiotic residues has been regarded as an important route of ARGs entering into the soil environment (Zhu et al. 2013; Chen et al. 2016; Zhang et al. 2017; Gou et al. 2018). However, the selection pressure imposed by antibiotics is not long-lasting owing to the rapid degradation of antibiotics in soil (Hu et al. 2016a). There is increasing awareness that a wide array of other factors, in addition to antibiotics, may play an important role in the evolution and environmental spread of antibiotic resistance (Hu et al. 2017). In particular, heavy metals, with an estimated half-life ranging for hundreds of years, may function as a strong selection agent for antibiotic resistance and metal resistance among bacteria through the common mechanisms of co-resistance (physical genetic linkage between different genes), cross-resistance (single gene encoding resistance to multiple antibiotics and metals) and co-regulation (shared regulatory system to antibiotic and metal resistance) (Berg et al. 2005; Baker-Austin et al. 2006). A few studies have postulated the co-selection mechanisms as a major pathway for the elevated frequencies of antibiotic resistance in heavy-

metal contaminated environmental settings (Ji et al. 2012; Hu et al. 2017; Zhang et al. 2018).

Heavy metals such as copper (Cu) are commonly utilized as animal feed to control disease and promote growth and can be introduced to soils following land amendment of metal-containing animal wastes, fertilizers and sewage sludge (Seiler and Berendonk 2012). Although Cu is an essential element for various cellular components and physiological functions of the bacterial cells, a high concentration of Cu can be toxic to soil organisms via causing damage to nucleic acids and cellular membranes (Knapp et al. 2011; Seiler and Berendonk 2012). Previous studies have documented the long-term impact of Cu contamination on the occurrence of antibiotic resistance and reported the linkage between Cu pollution and the increasing occurrence of ARGs in soils (Hu et al. 2016b; Poole 2017). It was found that Cu had stronger impact on the patterns of ARGs compared with other types of heavy metals such as lead, nickel and zinc (Knapp et al. 2011; Zhu et al. 2013). Meanwhile, most of previous studies of the long-term metal-induced development of ARGs were conducted in soils contaminated with multiple antibiotics, metals and other antimicrobial agents, which hinders our ability to infer how Cu exposure directly contributes to the evolution of antibiotic resistance. To date, no studies have explored the temporal dynamics of copper resistance genes (CRGs) and a wide spectrum of ARGs in agricultural soils exposed to short-term Cu contamination, and it remains largely unknown whether the short-term Cu exposure can select for both antibiotic and copper resistance. Such information is of great value for us to understand the behaviors of soil bacterial communities under the selection pressure which enables a better prediction of the evolution of ARGs in the environment.

This study aimed to examine the effects of short-term Cu exposure on the abundance, diversity, and horizontal transfer potential of 285 ARGs, 10 MGEs and two CRGs (the *copA* and *pcoR* genes) in laboratory soil microcosms amended with a gradient of Cu concentrations using the high-throughput quantitative PCR (HT-qPCR). We explored the possibility that short-term Cu exposure may lead to the co-selection of antibiotic and metal resistance due to the possible genetic linkage of these resistance genes on MGEs. The following hypotheses were tested: (1) Cu amendment can enhance the abundance and diversity of soil ARGs, which are positively correlated with the Cu concentrations; (2) Cu amendment can increase the abundance of CRGs, which might show a similar temporal pattern to that of ARGs; and (3)

The relative abundances of ARGs, MGEs and CRGs can be positively correlated if these resistance genes are located on the same MGEs or in the same bacterial hosts.

Materials and Methods

Soil sampling

Soil samples used for construction of the laboratory microcosm experiment were collected from a long-term research vegetable farm at Clyde (38°07'S, 145°19'E), Victoria, Australia. Mean annual temperature at this site is 19.4 °C, and mean annual precipitation is 819 mm. This vegetable farm has been applied with only inorganic fertilizers during the preceding five years without known history of organic fertilizer application. The soil is characterized as a Loamy Sand with a pH (H_2O) value of 6.7. We collected the surface soils (0-10cm) and transported them to the laboratory on ice, passed through 2 mm mesh, and archived at 4 °C prior to the start of the incubation. Details on the soil properties are given in Table 1.

Soil microcosms and DNA extraction

We established six treatments in 250 ml vials with three replicates containing 20 g of fresh soils: untreated soil samples (control), and treatments with 50, 100, 250, 500, 1000 mg Cu kg^{-1} soil (Cu50, Cu100, Cu250, Cu500 and Cu1000, respectively). The $CuCl_2$ solution was added to the soil microcosms to reach the corresponding Cu concentrations. All the soil microcosms were maintained at 60% of the water filled pore space through regular replenishment of sterilized water. The aerobic condition was maintained through refreshing air in the vials twice a week. A total of 126 soil microcosms were incubated in the dark at 25°C, and destructively sampled at days 0, 7, 14, 21, 28, 42 and 56. The DNeasy PowerSoil Kit (QIAGEN INC., Germantown, MD, USA) was used to isolate microbial genomic DNA from the soil samples following the manufacturer's instructions.

High-throughput qPCR (HT- qPCR) analysis of ARGs and MGEs

The Wafergen SmartChip Real-time PCR platform (WaferGen Biosystems, Fremont, CA, USA) was used for the high-throughput qualification of ARGs and MGEs in soils as previously described (Su et al. 2015; Gou et al. 2018). A total of 285 probes targeting all the major categories of antibiotic resistance and 10 probes targeting MGEs were included in the

current version of the HT-qPCR array (Looft et al. 2012; Johnson et al. 2016). The 100 nl qPCR reaction mixture contained the SensiMix SYBR No-ROX reagent, primers and genomic DNA. The qPCR reaction was run in triplicate for each sample using the amplification conditions: 10 min at 95°C, followed by 40 cycles of 30 s at 95°C and 30 s at 60°C. The HT-qPCR results were quality-filtered according to the criteria as described previously (Su et al. 2015; Hu et al. 2017) and the relative abundances of ARGs and MGEs (as compared to 16S rRNA gene) were calculated using the comparative C_T method (Schmittgen and Livak 2008).

Quantitative PCR analysis of copper resistant genes (CRGs) and 16S rRNA gene

The Bio-Rad CFX384™ Real-Time PCR Detection System (Bio-Rad, Herculers, CA, USA) was used to qualify the *copA* and *pcoD* genes using the primer pairs copAF2010/copAR2333 (Li et al. 2012) and pcoR1/pcoR2 (Trajanovska et al. 1997), respectively. The 10 µl PCR reaction mixture consisted of 5 µl SensiMix SYBR No-ROX reagent (Bioline), 0.5 µl of each primer (10 µM), 2 µl of 10-fold diluted DNA template, and 2 µl of nuclease-free water. The thermal-cycling conditions were: 95°C for 10 min, 40 cycles of 95°C for 30 s, 51°C for 30s, and 72°C for 45 s. The bacterial 16S rRNA gene was quantified using the primer pair BACT1369F/PROK1492R (Suzuki et al. 2000). Amplification conditions were: 95°C for 10 s, 35 cycles of 95°C for 15 s and 56°C for 1 min. The CRGs (i.e. the *copA* and *pcoD* genes) and the bacterial 16S rRNA gene were PCR-amplified from the soil DNA, linked to the pGEM®-T Easy Vectors, and transferred to JM109 High Efficiency Competent Cells (Promega, Madison, USA). Plasmid DNA was extracted from positive clones using the PureYield™ Plasmid Miniprep System (Promega) and sequenced to confirm their identities. Standard curves were prepared from 10-fold serial dilutions of plasmids with inserts of the targeted gene sequences. We conducted melting curve analysis after each qPCR run to verify the specificity of PCR amplicons.

Network analysis and visualization

The CoNet Cytoscape plug-in method was employed to explore the co-occurrence patterns among the detected ARGs, MGEs and CRGs in all the samples as previously described (Soffer et al. 2015; Hu et al. 2017). The pairwise interaction scores were calculated with Pearson and Spearman correlation methods and Kullback-Leibler and Bray-Curtis

dissimilarity methods. The potential false-positive correlations were eliminated by the conduction of the ReBoot procedure with 100 permutations and inclusion of ARGs with an occurrence of > 30 across all the samples. We merged the resultant P-values using the Brown method. The significant pairwise correlations (with a correlation coefficient above 0.6 and a significance level below 0.05) were utilized to construct the co-occurrence network and visualized using the Fruchterman Reingold algorithm on the interactive Gephi platform (Bastian et al. 2009).

Statistical analysis

Significant differences ($P < 0.05$) in the relative and absolute abundance of ARGs, MGEs and CRGs across treatments were detected using the one-way analysis of variance (ANOVA) test after correction for multiple testing. Pearson correlation test was performed to assess the correlations between the abundances of ARGs, MGEs, and CRGs. Non-metric multidimensional scaling (NMDS) analysis was conducted to visualize the differences in the relative abundance of ARGs across different treatments based on the Bray-Curtis dissimilarity matrices. The effects of Cu-amended treatments on the ARG patterns were tested by performing the permutational multivariate analysis of variance (PerMANOVA) using the 'vegan' package of the R software with 999 permutations.

Results and discussion

Copper amendment increased the diversity and abundance of ARGs, MGEs and CRGs

While many studies have examined the impacts of heavy metals on soil ARGs in long-term experimental sites, less is known about the short-term dynamics of ARGs and MGEs following Cu amendment to soils. In this study, the HT-qPCR array was employed to assess the abundance and diversity of ARGs and MGEs in an agricultural soil treated with different Cu concentrations (0~1000 mg kg^{-1}) in laboratory microcosms. A total of 126 unique ARGs and 6 MGEs were detected across all the samples. In line with previous profiling of ARGs in animal farms (Zhu et al. 2013), manure-contaminated soils (Hu et al. 2016a), antibiotics-treated soils (Zhang et al. 2017), and human-impacted estuaries (Zhu et al. 2017), the detected ARGs in this study could encode resistance to all the major categories of common antibiotics, with the most frequent types being multidrug (24.3%), aminoglycoside (19.9%), tetracycline

(14.4%), and β-lactam (10.9%) (Fig. 1A). The detected ARGs were classified into different resistance mechanisms including efflux pump (41.6%), antibiotic deactivation (38.3%) and cellular protection (18.9 %) (Fig. 1B). The average numbers of detected ARGs highly varied across the different Cu-amended treatments, with the highest values recorded in the Cu50 treatment (Fig. 1C). The diversity of ARGs in the high-level Cu treatments (Cu250, Cu500 and Cu1000) was significantly lower than that in the Cu50 treatment ($P < 0.05$). The diversity of MGEs also significantly changed across the different Cu-treated treatments (Fig. 1D).

In contrast to the diversity patterns, the absolute abundance and relative abundance (as compared to the bacterial 16S rRNA gene) of ARGs and MGEs constantly increased with the increasing Cu concentrations (Fig. 2). The highest abundance of ARGs and MGEs was observed in Cu1000 and significantly higher than that in untreated soils and low-level Cu treatments (Cu50 and Cu100) ($P < 0.05$). In particular, the abundances of genes encoding resistance to multidrug, aminoglycoside and sulfonamide significantly increased with the increasing Cu concentrations (Fig. 2A, 2C). The NMDS analysis of the relative abundance of individual ARG subtypes based on the Bray-Curtis dissimilarity matrices revealed that ARG profiles gradually shifted across the gradient of Cu contamination (Fig. 3), which revealed that Cu contamination was a major determinant shaping the ARG patterns (PerMANOVA, $P < 0.05$). The absolute and relative abundances of the *copA* and *pcoR* genes exhibited different patterns from that of ARGs and MGEs along the gradient of Cu concentration (Fig. 4). The abundance of the *copA* gene was significantly higher than that of the *pcoR* gene across all the treatments ($P < 0.05$). The absolute abundance of the *copA* gene was found to be highest in the Cu100 treatment, which was significantly higher than that in other treatments ($P < 0.05$) (Fig. 4C). The *pcoR* gene showed the similar variation tendency, but with the highest values recorded in the Cu50 treatment (Fig. 4B, 4D).

Different from the previous studies of the long-term impacts of Cu contamination on the distribution patterns ARGs (Berg et al. 2005; Hu et al. 2016b), our results showed that even a short-term Cu exposure can significantly enhance the abundance of antibiotic resistance and influence the compositions of soil ARGs. This finding suggested that soil bacterial communities can quickly evolve under the metal stress and develop resistance to Cu and antibiotics in a short term of two months. In line with our results, previous studies reported

that freshwater bacterial communities exposed to heavy metal contamination can lead to increased levels of multi-resistant microorganisms (Stepanauskas et al. 2006), and long-term heavy metal (e.g., copper and nickel) exposure can induce bacterial adaptations that result in increased levels of resistance to multiple antibiotics in soils (Berg et al. 2005; Knapp et al. 2011; Hu et al. 2016b, 2017). Heavy metal elements in swine feed had strong correlations with the occurrence and abundances of multidrug-resistant bacteria (Zhu et al. 2013; Medardus et al. 2014). Our study provided further evidence that soil resident bacteria are highly adaptable and can rapidly evolve resistance to the added heavy metals through the development of both antibiotic and metal resistance. However, it was found that the abundances of ARGs and CRGs showed different responses to Cu amendment, for example, ARG abundances increased with increasing Cu concentrations (Fig. 2) while CRG abundances showed a general declining trend along the gradient of Cu concentrations (Fig. 4). This suggests that the targeted ARGs and CRGs might reside in different bacterial hosts, and ARG-bearing bacteria and CRG-bearing bacteria might have different preference for Cu concentrations, with ARG-bearing bacteria favoured at high Cu concentrations while CRG-bearing bacteria favoured at low Cu concentrations. Therefore, given the anthropogenic levels of metal contamination in the environment, it is assumed that metal pollution can exert a strong selection pressure for antibiotic resistance, but antibiotic resistance and metal resistance are not necessarily positively associated under the Cu stress.

Temporal changes of ARGs, MGEs and CRGs

The temporal changes of ARGs, MGEs and CRGs in the soil microcosms were explored at seven time points (days 0, 7,14, 21, 28, 42 and 56). Owing to the very similar temporal patterns of ARGs, MGEs and CRGs in different Cu-amended treatment, only the treatments with the highest gene abundances were compared to the control treatment (Fig. 5). The absolute abundances of total ARGs (Fig. 5A) and MGEs (Fig. 5B) increased shortly following the Cu addition, reached the highest level at day 14, and then gradually declined over time during the incubation. Similarly, the absolute abundances of the *copA* and *pcoR* genes (Fig. 5C, 5D) in Cu-amended soils climbed to the highest level at day 14 and showed the similar declining patterns to that of ARGs and MGEs. By contrast, the absolute abundances of ARGs, MGEs and CRGs in the control treatment remained largely unchanged during the short-term

microcosm incubation. Our results suggested that soil resident microorganisms can rapidly develop resistance to the Cu stress in less than two weeks possibly through the evolution of antibiotic resistance and copper resistance. However, exposure of soil resident bacteria to heavy metals for more than two weeks in soil microcosms can be toxic and damage cellular membranes and genomic DNA, hence, sensitive bacterial hosts could be killed, resulting in the reduction of ARGs (Burch et al. 2013) and CRGs (Wang et al. 2016). The reduction of ARGs/CRGs over time following after day14 can be also attributed to the gradual out-competition of ARGs/CRGs-bearing bacteria by the soil resident microbiomes, as the ability of resistance to antibiotics/metals can be an additional energy cost of bacterial hosts (Martinez 2009), which can become less competitive when nutrients are becoming limited in a soil microcosm. However, it should be noted that the level of antibiotic resistance is still significantly higher than the background level in the control treatment, suggesting the enrichment of ARGs during to short-term Cu exposure can be long-standing in soils. This finding might help explain the phenomenon that despite tremendous efforts to reduce the global consumption of antibiotics, there is increasing prevalence of ARGs in environmental settings (Li et al. 2017).

Relationships among ARGs, MGEs and CRGs

Pearson correlation analysis demonstrated that the relative abundances of total ARGs and specific categories of ARGs (including aminoglycoside, FCA and sulfonamide resistance) had significantly positive correlation with that of total transposase and intergrase genes ($P < 0.05$, Table 2). Moreover, the MLSB (Macrolide-Lincosamide-Streptogramin B), multidrug and tetracycline resistance genes had also significantly positive relationships with integrase genes ($P < 0.05$, Table 2). This finding was further supported by the co-occurrence network analysis which revealed intensive correlations between ARGs and MGEs (Fig. 6). The resultant network included 23 nodes (ARG or MGE subtypes) and 80 edges which could be separated into three modules. The *tnpA-05* gene (belonging to MGEs) was intensively connected with multiple genes encoding potential resistance to aminoglycoside, multidrug, FCA and sulfonamide, indicating that these co-occurring ARGs have a likelihood of dispersal to other bacteria and pathogens through the mechanism of horizontal gene transfer (HGT). It has been well-documented that some transposons harbor metal-resistance operons and

integrons containing multiple ARGs (Baker-Austin et al. 2006) and might play important roles in the horizontal transfer of antibiotic and metal resistance genes in environmental microbial populations (Gillings et al. 2015). Although a range of biotic and abiotic factors (e.g. soil pH, temperature, soil moisture contents, soil types, and vegetation) can affect the frequencies of HGT of ARGs in soils (Aminov 2011), our results indicated the HGT potential of multiple antibiotic resistance in bacterial communities of Cu-contaminated soils. In addition, it should be noted that the network analysis can only provide correlative evidence for the possible links between ARGs and MGEs, which requires further validation experiment to confirm their connections in bacterial hosts.

In contrast to the previous reports about the co-occurrence of CRGs and ARGs under the Cu stress, for example the co-occurrence of *pcoA* with *erm*(A) and *intl*1 (Besaury et al. 2016), co-transmission of *tcrB*/*cueO* with *erm*(B) in *Enterococcus* and *Enterococci* (Amachawadi et al. 2013; Silveira et al. 2014), no significant correlation was found between the relative abundances of ARGs and the *pcoR* gene, and the *copA* gene had significantly negative relationships with the aminoglycoside, FCA, sulfonamide resistance genes and MGEs (Table 3). These results suggested that the targeted *copA* and *pcoR* genes and the detected ARGs may be not intimately linked in the same gene cassettes or these genes might reside in different bacterial strains. Similarly, previous studies did not find the co-occurrence of the *pcoA* gene with ARGs in *Salmonella* (Yin et al. 2017), and low co-occurrence frequency between ARGs and CRGs (including the *copA* gene) was observed on plasmids under the stress of both antibiotics and metals (Pal et al. 2015). These findings suggest that bacteria resistant to antibiotics and those resistant to Cu might belong to different groups, and no co-selection is actually occurring in this short-term microcosm.

Conclusions

To be able to prevent further development of environmental resistome, it is important to understand how bacterial antibiotic resistance evolves in the environment. Our study provided empirical evidence that even short-term exposure to copper can contribute to the development of bacterial antibiotic resistance in agricultural soils. The changes in antibiotic resistance are not significantly and positively associated with that of metal resistance, indicating that co-

selection might be not the main mechanism for the evolution of antibiotic and metal resistance in short-term Cu-contaminated soil microcosms. Moreover, the transposase genes had strong and positive relations with ARGs during the incubation, suggesting the HGT potential of ARGs might be enhanced by the Cu exposure. Together, our findings caution that the heavy metals such as Cu widely present in the soil environment can lead to a high degree of antibiotic resistance and may contribute to the dissemination of environmental resistomes.

Acknowledgments

This study was supported by Australian Research Council (DP170103628) and Australian-China Joint Research Centre (ACSRF48165).

Conflict of Interest: The authors declare that they have no conflict of interest.

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460 Zhu YG, Zhao Y, Li B, Huang CL, Zhang SY, Yu S et al (2017) Continental-scale pollution of
461 estuaries with antibiotic resistance genes. Nat Microbiol 2: 16270
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463

464 **Table 1** The basic properties of the arable soil used in this study

pH	Color	Texture	Organic matter (%)	Organic carbon (%)	Total carbon (%)	Total nitrogen (%)	Nitrate (mg kg^{-1})	Ammonium (mg kg^{-1})
6.7	grey	Sandy loam	3.8	2.2	2.4	0.27	240	19
C:N ratio	**Sulphur (mg kg^{-1})**	**Phosphorus (mg kg^{-1})**	**Available potassium (mg kg^{-1})**	**Calcium (cmol kg^{-1})**	**Magnesium (cmol kg^{-1})**	**Aluminium (mg kg^{-1})**	**Copper (mg kg^{-1})**	**Zinc (mg kg^{-1})**
8.9	39	620	220	8.4	2.6	<9.0	12	24

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Table 2 Pearson correlations of the relative abundances of ARGs and MGEs across all the soil samples

	Relative abundances of MGEs	
	Transposase	Integrase
Total ARGs	**0.599 (<0.001)**	**0.611 (<0.001)**
Aminoglycoside	**0.820 (<0.001)**	**0.606 (<0.001)**
β-lactam	0.088 (0.380)	0.178 (0.260)
FCA	**0.810 (<0.001)**	**0.446 (0.003)**
MLSB	0.084 (0.397)	**0.323 (0.037)**
Multidrug	0.143 (0.295)	**0.402 (0.008)**
Sulfonamide	**0.853 (<0.001)**	**0.516 (<0.001)**
Tetracycline	0.027 (0.564)	**0.392 (0.010)**
Vancomycin	0.102 (0.319)	0.243 (0.120)
Other	0.051 (0.550)	0.279 (0.073)

Values showed are the R-values obtained from the Pearson correlation test, and values showed in the brackets are the corresponding *P*-values. Significant correlations ($P < 0.05$) are highlighted in bold numbers. (MLSB: Macrolide-Lincosamide-Streptogramin B resistance genes; FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes)

Table 3 Pearson correlations of the relative abundance between CRGs (*copA* and *pcoR*) and ARGs and MGEs across all the samples

	Gene copies /16S rRNA copies	
	copA	*pcoR*
Aminoglycoside	**-0.529 (< 0.001)**	-0.179 (0.257)
Beta Lactamase	-0.069 (0.662)	0.123 (0.438)
FCA	**-0.635 (< 0.001)**	-0.151 (0.339)
MLSB	-0.097 (0.541)	-0.262 (0.093)
Multidrug	-0.060 (0.704)	-0.074 (0.643)
Sulfonamide	**-0.591 (< 0.001)**	-0.156 (0.323)
Tetracycline	-0.084 (0.598)	-0.272 (0.081)
Vancomycin	-0.085 (0.592)	0.158 (0.316)
other	0.041 (0.798)	-0.025 (0.877)
Transposase	**-0.545 (< 0.001)**	-0.222 (0.158)
Integrase	**-0.337 (0.029)**	-0.137 (0.386)

Values showed are the R-values obtained from the Pearson correlation test, and values showed in the brackets are the corresponding *P*-values. Significant correlations ($P < 0.05$) are highlighted in bold numbers.

Figure legends

Figure 1 Classification of all the detected ARGs based on the classes of antibiotics (A); Classification of all the detected ARGs based on the resistance mechanisms (B); The number of ARGs (C) and MGEs (D) detected across different Cu-contaminated treatments in the agricultural soil. Different letters above the columns represent a significant difference ($P < 0.05$). (MLSB: Macrolide-Lincosamide-Streptogramin B resistance genes; FCA: fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes)

Figure 2 The relative and absolute abundance of ARGs (A and C) and MGEs (B and D) detected across all the samples. Different letters above the columns represent a significant difference ($P < 0.05$).

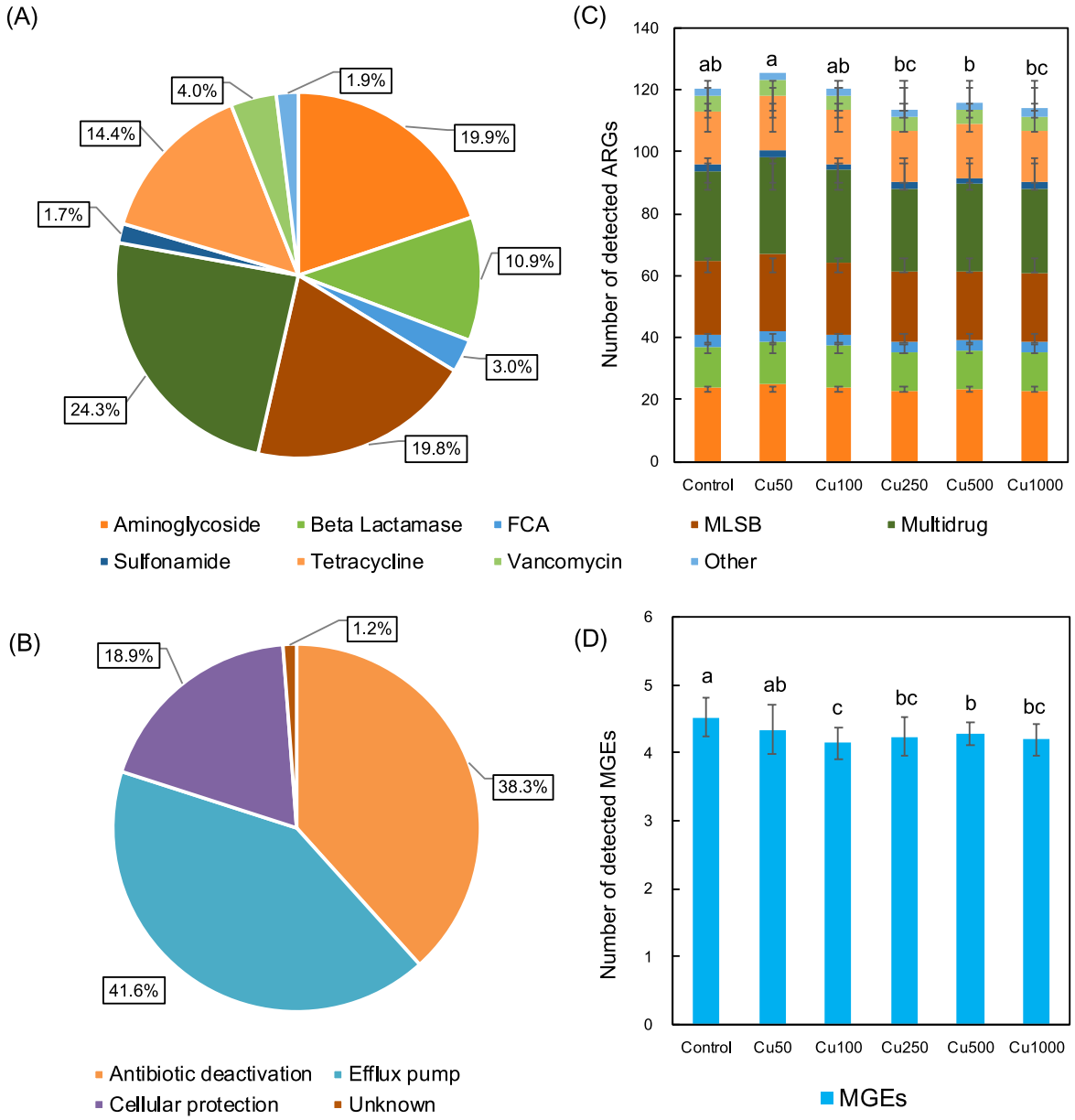
Figure 3 The non-metric multidimensional scaling (NMDS) ordinations showing the Bray-Curtis dissimilarity of the relative abundance of ARGs in all samples along the gradient of Cu concentrations. The 2D stress value is 0.12, which indicates that these data could be well represented by the 2D ordination.

Figure 4 The relative and absolute abundance of copper resistance genes *copA* (A and C) and *pcoR* (B and D). Different letters above the columns represent a significant difference.

Figure 5 The temporal changes of the absolute abundance of ARGs (A) and MGEs (B) as well as the *copA* (C) and *pcoR* (D) genes in the control and Cu-contaminated treatments during the incubation.

Figure 6 The co-occurrence patterns among the detected ARGs and MGEs in all the soils as revealed by the network analysis. Different modularity classes are represented by the nodes with different colors, and strong and significant correlations between nodes are represented by the edges.

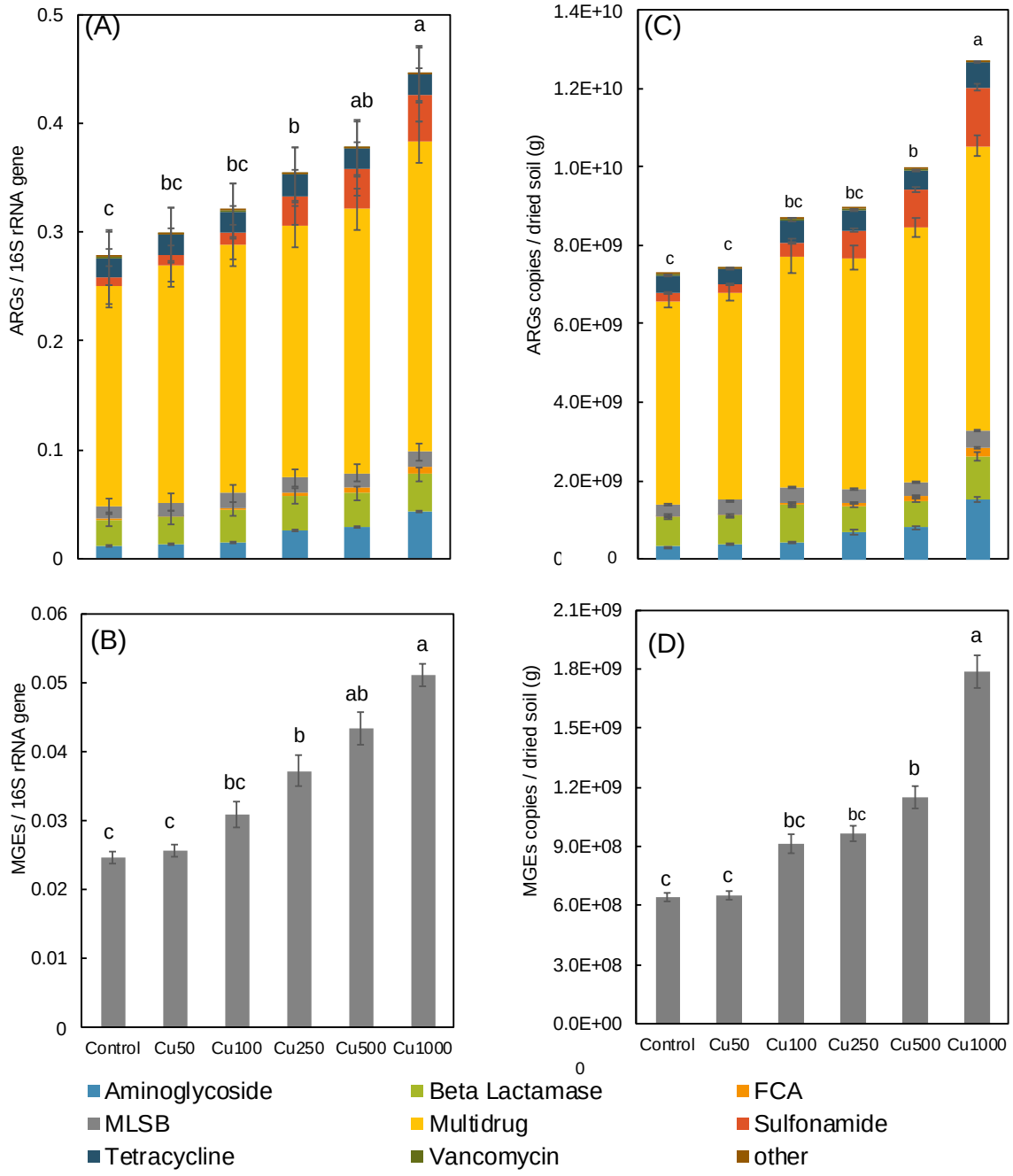
508 **Figure 1**



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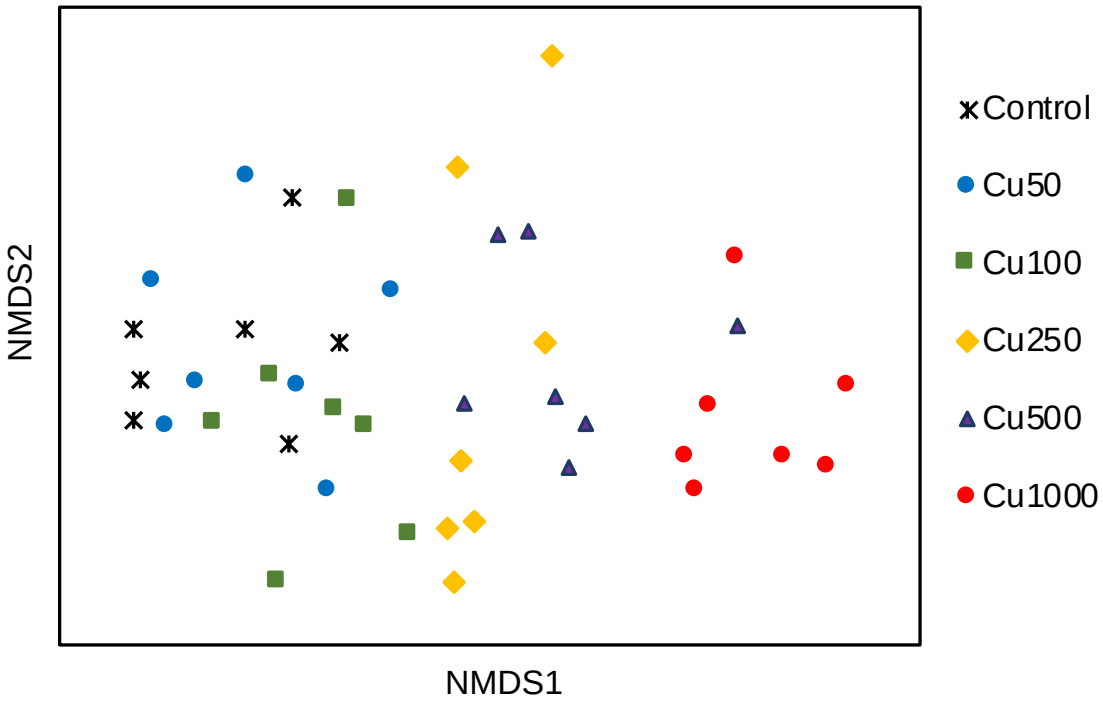
511 **Figure 2**



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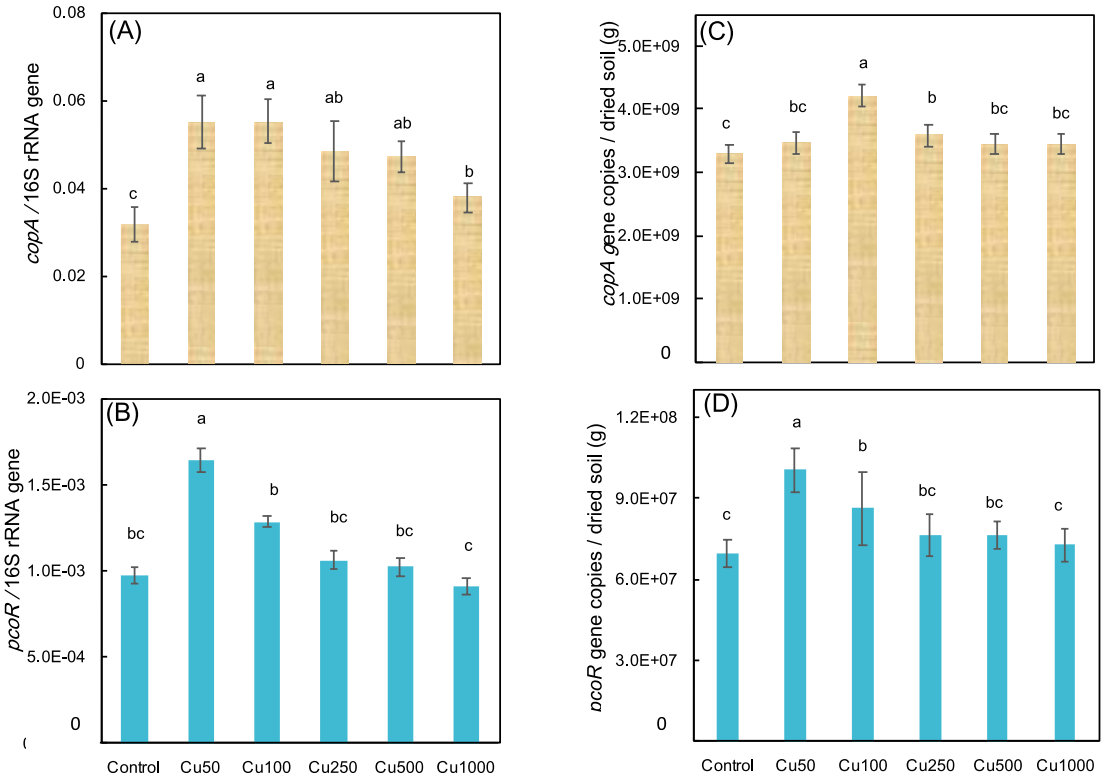
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514 **Figure 3**



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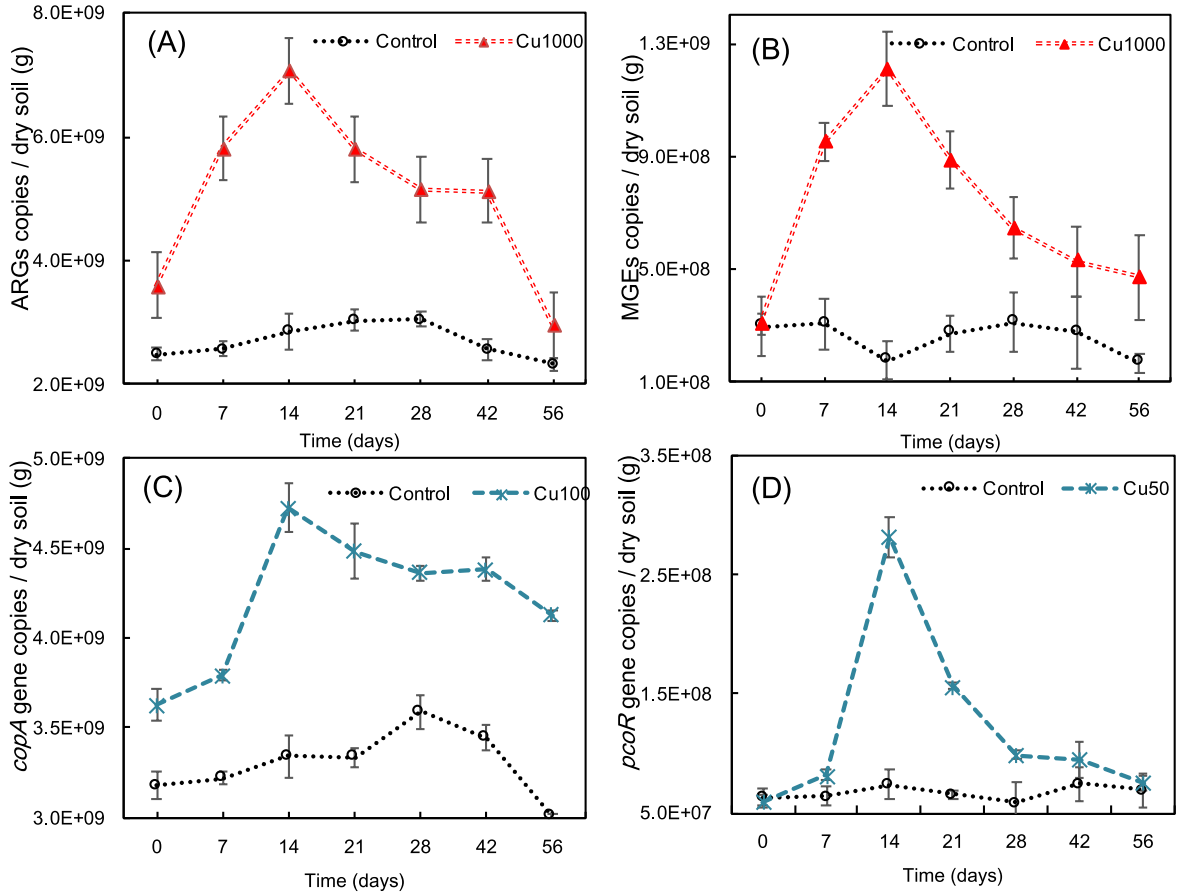
517 **Figure 4**



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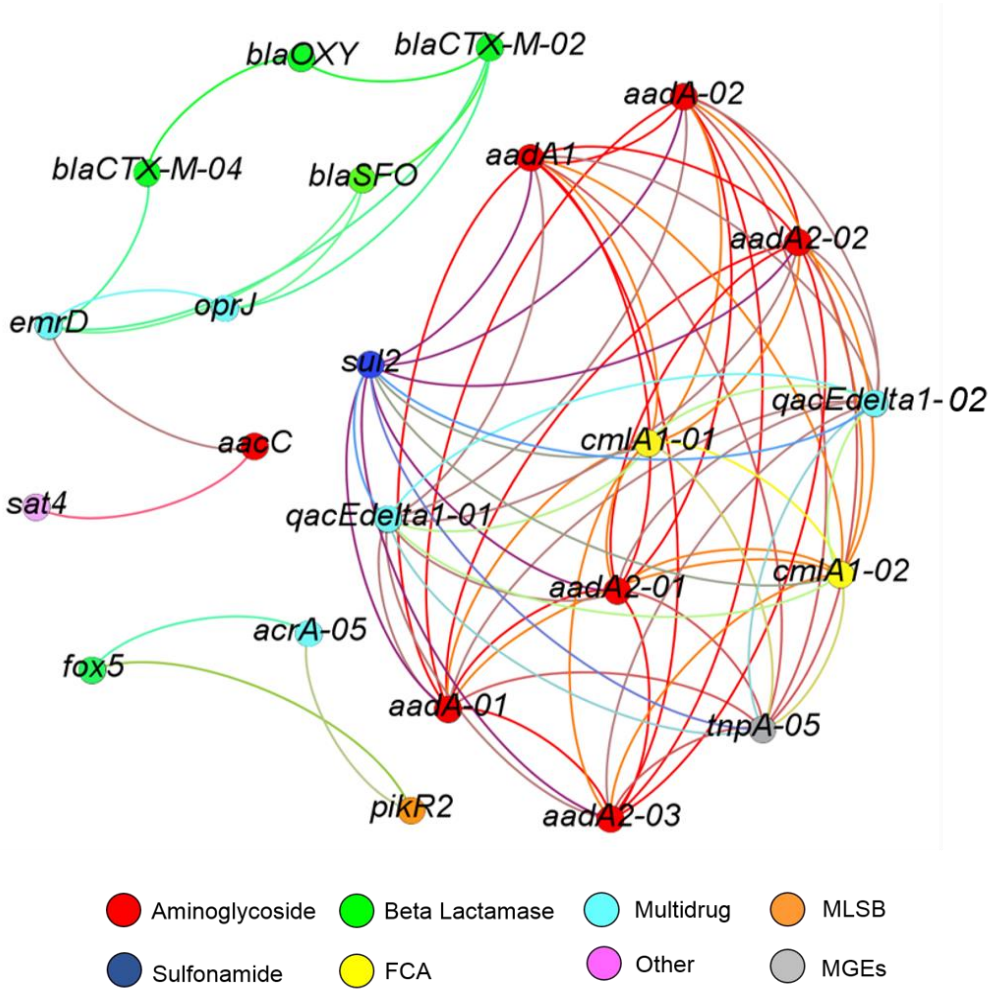
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520 **Figure 5**



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522 **Figure 6**



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