Antibiotic-Selected Gene Amplification Heightens Metal Resistance

David A. Hufnagel,a,b,c Jacob E. Choby,a,b,c Samantha Hao,d Anders F. Johnson,a Ⓡ Eileen M. Burd,a,c,f Charles Langelier,d,g David S. Weiss,a,b,c,h

aEmory Antibiotic Resistance Center, Atlanta, Georgia, USA
bEmory Vaccine Center, Atlanta, Georgia, USA
cDivision of Infectious Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, USA
dDivision of Infectious Diseases, Department of Medicine, University of California, San Francisco, California, USA
eDepartment of Microbiology and Immunology, Emory University, Atlanta, Georgia, USA
fDepartment of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia, USA
gChan Zuckerberg Biohub, San Francisco, California, USA
hResearch Service, Atlanta VA Medical Center, Decatur, Georgia, USA

ABSTRACT The increasing frequency of antibiotic resistance poses myriad challenges to modern medicine. Environmental survival of multidrug-resistant bacteria in health care facilities, including hospitals, creates reservoirs for transmission of these difficult to treat pathogens. To prevent bacterial colonization, these facilities deploy an array of infection control measures, including bactericidal metals on surfaces, as well as implanted devices. Although antibiotics are routinely used in these health care environments, it is unknown whether and how antibiotic exposure affects metal resistance. We identified a multidrug-resistant Enterobacter clinical isolate that displayed heteroresistance to the antibiotic colistin, where only a minor fraction of cells within the population resist the drug. When this isolate was grown in the presence of colistin, a 9-kb DNA region was duplicated in the surviving resistant subpopulation, but surprisingly, was not required for colistin heteroresistance. Instead, the amplified region included a three-gene locus (ncrABC) that conferred resistance to the bactericidal metal, nickel. ncrABC expression alone was sufficient to confer nickel resistance to E. coli K-12. Due to its selection for the colistin-resistant subpopulation harboring the duplicated 9-kb region that includes ncrABC, colistin treatment led to enhanced nickel resistance. Taken together, these data suggest that the use of antibiotics may inadvertently promote enhanced resistance to antimicrobial metals, with potentially profound implications for bacterial colonization and transmission in the health care environment.

IMPORTANCE To inhibit bacterial transmission and infection, health care facilities use bactericidal metal coatings to prevent colonization of surfaces and implanted devices. In these environments, antibiotics are commonly used, but their effect on metal resistance is unclear. The data described here reveal that exposure of a human isolate of Enterobacter cloacae to a last-line antibiotic, colistin, resulted in a DNA amplification that does not confer antibiotic resistance but instead facilitates resistance to the toxic metal nickel. This highlights a novel aspect of antibiotic and metal interplay. Concerningly, these data suggest the use of antibiotics could in some cases promote bacterial survival and colonization in the health care environment and ultimately increase transmission and infection of patients.

KEYWORDS Enterobacter, colistin, gene amplification, heteroresistance, metal resistance, nickel

Citation Hufnagel DA, Choby JE, Hao S, Johnson AF, Burd EM, Langelier C, Weiss DS. 2021. Antibiotic-selected gene amplification heightens metal resistance. mBio 12:e02994-20. https://doi.org/10.1128/mBio.02994-20.

Invited Editor Bryan W. Davies, University of Texas at Austin

Editor Marvin Whiteley, Georgia Institute of Technology School of Biological Sciences

Copyright © 2021 Hufnagel et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Address correspondence to David S. Weiss, david.weiss@emory.edu.

Received 21 October 2020
Accepted 30 November 2020
Published 19 January 2021
Increasing resistance to antibiotics is a threat to modern medicine, in some cases precluding the treatment of bacterial infections and routine procedures that rely on these drugs such as surgeries, cancer chemotherapy, and organ transplantations. One prediction suggests that 10 million people will die worldwide each year from antibiotic-resistant infections by 2050 (1). In the United States alone, there are currently estimated to be over 150,000 annual deaths due to antibiotic-resistant bacteria (2). Infections caused by the carbapenem-resistant *Enterobacterales* (CRE; including *Enterobacter*, *Escherichia*, and *Klebsiella*) are one of the most worrisome threats since they increasingly require treatment with last-line antibiotics such as colistin and in certain cases are resistant to all available drugs (3).

*Enterobacterales* can persist in the environment for months, including in hospitals, which increases the opportunities for transmission of these pathogens to new hosts (4, 5). In fact, there are more than 700,000 health care associated infections each year in the United States, many of which are likely linked to the ability of bacteria to persist in the environment (6). Astoundingly, one study found that 92% of patients with a CRE infection had in the past year visited a health care facility (75.1% had acute care hospitalization), where rampant antibiotic use routinely selects for highly resistant bacteria (6, 7). To curb transmission of such pathogens to hospitalized patients, antimicrobial metals are utilized on patient contact surfaces and medical devices (8, 9). While other studies have demonstrated that metal exposure can induce antibiotic resistance, it is unclear whether antibiotics affect bacterial metal resistance and thus capacity to colonize antimicrobial metal surfaces.

In studying human clinical isolates of *Enterobacter cloacae* from the health care setting, we identified a strain (“R/S”) that exhibits heteroresistance to colistin (10, 11). Heteroresistance is a phenomenon where a minor group of cells in a population are resistant to an antibiotic and coexist with a majority susceptible population (12). The growth of R/S in colistin (100 μg/ml in Mueller-Hinton medium [MH]) selected for the resistant subpopulation; however, subsequent drug-free passage resulted in a return to the baseline frequency of 10% resistant cells (see Fig. S1A in the supplemental material) (11). Analysis of mapped reads from Illumina whole-genome sequencing of R/S grown in the presence or absence of colistin revealed identical sequences with no genetic differences (11). However, further analysis of gene copy number revealed a single 9-kb region that was duplicated exclusively when R/S was grown in the presence of colistin (see Fig. S1B). Interestingly, this 9-kb region is flanked by transposon-related elements, suggesting gene amplification might rely on these sequences, as has been observed for antibiotic resistance genes and a nickel resistance locus in *Pseudomonas* (13, 14). Using quantitative PCR (qPCR) on genomic DNA isolated from R/S grown with or without colistin, we confirmed the duplication of the 9-kb region in colistin-treated cultures (Fig. 1A). Subsequent overnight passage in the absence of colistin resulted in reversion to a single copy of the 9-kb region and reexposure of the passaged strain to colistin led to its reamplification (Fig. 1A), correlating with the colistin resistance dynamics displayed by R/S (see Fig. S1A) (11). In contrast, when R/S was challenged with 0.25 × MIC of other antibiotics (amikacin, cefepime, or ciprofloxacin), we observed no duplication of the 9-kb region, highlighting the specificity of this selection (see Fig. S1C).

To investigate the potential role of the 9-kb region in colistin heteroresistance, we used lambda red mutagenesis (15, 16) to generate an R/S isogenic mutant completely lacking the 9-kb region (Δ9kb). Surprisingly, R/S and Δ9kb had a similar frequency of resistant colonies growing in the zone of inhibition when plated with a colistin MIC test gradient strip (0.5 McFarland was used for spread plating [Liofilchem, Waltham, MA]), indicating the 9-kb region was not required for colistin heteroresistance (Fig. 1B). Similarly, population analysis profile, which is used to quantify bacterial subpopulations via serial dilution and plating on doubling dilutions of antibiotic to determine the percent survival (12), revealed no difference in the frequency of the colistin-resistant subpopulation between R/S and Δ9kb (Fig. 1C). Finally, treatment of R/S and Δ9kb
with colistin in broth indicated that each strain harbored a resistant subpopulation that was able to rapidly expand in the antibiotic (Fig. 1D). Taken together, these data show that despite the amplification of the 9-kb region during colistin exposure, the corresponding genes do not contribute to colistin heteroresistance.

Since the 9-kb region did not affect colistin resistance, we next determined its physiological role. While there were no known colistin resistance genes present within this region, bioinformatic analysis revealed that it contained multiple putative metal resistance genes (Fig. 2A). We therefore quantified the MIC of R/S and $D_{9\text{kb}}$ to a panel of metals via broth microdilution (BMD) (Fig. 2B). Briefly, $5 \times 10^{8}$ CFU of bacteria were inoculated into a 96-well plate with doubling dilutions of metals and incubated for 20 h at 37°C. Of the metals tested, the 9-kb mutation only reduced the MIC of R/S to nickel [nickel(II) sulfate; Alfa Aesar, Ward Hill, MA] (Fig. 2B). Disk diffusion (6-mm paper discs; BBL, Sparks, MD) similarly indicated an increased susceptibility of $D_{9\text{kb}}$ compared to R/S (Fig. 2C).

To identify genes within the 9-kb region that confer enhanced resistance to nickel, we mutated two putative metal resistance operons containing major facilitator superfamily (MFS) genes (Fig. 2A). Disk diffusion analysis revealed that one of the putative operon mutants phenocopied the increased nickel susceptibility of $D_{9\text{kb}}$, and the deleted genes were termed $ncrABC$ based on high sequence similarity (100% amino acid and 99% nucleotide) to nickel resistance genes first described in *Leptospirillum ferriphilum* (17) (Fig. 2C). The second mutant lacking a putative operon ($D_{\text{operon2}}$) displayed a similar level of nickel resistance as R/S (Fig. 2C). In addition, the $D_{9\text{kb}}$ and $D_{ncrABC}$ strains had decreased survival on nickel agar plates compared to R/S, whereas $D_{\text{operon2}}$ phenocopied R/S (Fig. 2D). Both $D_{9\text{kb}}$ and $D_{ncrABC}$ strains had similar growth kinetics as R/S in MH, highlighting that the nickel susceptibility of these strains
FIG 2 Colistin-selected DNA duplication confers resistance to nickel. (A) Schematic of a 9-kb region duplicated in Enterobacter cloacae strain R/S upon growth in colistin as detected by genome sequencing and mapping of raw, unfiltered reads. MFS, major facilitator superfamily. (B) Broth microdilution of various metals on R/S and Δ9kb to determine MICs [Ni, nickel(II) sulfate; Ca, calcium chloride; Co, cobalt chloride; Cr, sodium chromate; Cu, copper(II) sulfate; Fe, iron(II) sulfate; Mg, magnesium chloride]. (C) Discs containing 1.5 mg of nickel(II) sulfate were added to 0.5 McFarland standard of R/S and strains lacking 9-kb, ncrABC, and operon2 spread plates. (D) R/S and isogenic mutants were plated with or without 8 mM nickel(II) sulfate to determine the percent survival of each isolate. (E) 10⁷ CFU of R/S and the indicated mutants were grown in 8 mM nickel(II) sulfate, and the surviving CFU were determined at the indicated time points. (F) E. coli K-12 BW25113/pBAV and pncrABC were plated with or without 4 or 8 mM nickel(II) sulfate to determine the percent survival of each isolate. (G) Nickel population analysis profile of R/S and ΔncrABC strains grown for 5 h in MH with or without 100 µg/ml colistin prior to CFU enumeration of plates containing various concentrations of nickel(II) sulfate. Significance values determined by using a Student two-tailed t test (*, P < 0.05; **, P < 0.01). For panels D, E, and G, the data shown are as means with the standard deviations for a single representative experiment with three biological replicates. For panel F, the data shown as means and standard deviations of two independent experiments, each with biological replicates.
was not due to an underlying fitness defect (see Fig. S1D). In addition, the ncrABC mutation did not affect the resistance of R/S to a panel of antibiotics as tested via Vitek 2 (bioMérieux, Marcy l’Étoile, France), indicating that the contribution of ncrABC is specific (see Table S1 in the supplemental material). Importantly, in trans complementation of the ΔncrABC (ΔncrABC/pncrABC) strain restored survival in the presence of nickel, while the ΔncrABC and Δ9kb strains were killed (Fig. 2E). Further, the ΔncrABC/pncrABC strain exhibited a restored nickel MIC of 16 mM by BMD, similar to R/S and compared to an MIC of 4 mM for the Δ9kb and ΔncrABC strains (see Fig. S2A). These data suggest the ncrABC genes are important for nickel resistance in R/S, despite the presence of a homolog of the RcnA nickel resistance protein (59% amino acid identity to E. coli K-12 RcnA) encoded elsewhere in the genome. The ΔncrABC/pncrABC strain also exhibited increased survival by nickel disk diffusion and had no effect on fitness in MH (see Fig. S2B and C). Disk diffusion on minimal media similarly revealed that ncrABC deletion most robustly decreased nickel resistance (see Fig. S2D). In contrast, ncrABC deletion had no effect on chromium, copper, or iron resistance, but interestingly, cobalt resistance was slightly reduced in the ΔncrABC mutant (see Fig. S2D). These data clearly indicate that ncrABC mediates nickel resistance in R/S.

To determine whether ncrABC were sufficient for nickel resistance, we expressed these genes in E. coli K-12 and observed a >1,000-fold increase in survival in the presence of nickel (Fig. 2F). In addition, upon expression of ncrABC, the BMD nickel MIC of E. coli increased from 4 mM to 16 mM, the level observed for R/S (see Fig. S3). These data indicate that the ncrABC genes are sufficient to confer nickel resistance.

Since the ncrABC mutation confers nickel resistance and is encoded within a region of DNA that was duplicated upon colistin treatment, we hypothesized that colistin would lead to enhanced nickel resistance due to increased ncrABC gene dosage. Indeed, we observed an ncrABC-dependent increase in nickel resistance when R/S was grown with colistin (Fig. 2G). Importantly, genes in the 9-kb region still duplicated in the ΔncrABC mutant in the presence of colistin, indicating that the lack of colistin-dependent nickel resistance in the ΔncrABC strain was not due to an abrogation of amplification of this region (see Fig. S4). To determine whether a cationic molecule other than colistin would induce nickel resistance, we grew R/S in minimal media with or without iron (II) sulfate (+Fe) and assayed for survival in nickel and the nick zone of inhibition by disk diffusion assay (see Fig. S5A and B). While colistin increased nickel resistance in both assays, iron did not affect nickel resistance in R/S (see Fig. S5A and B).

We next determined whether colistin-induced nickel resistance was widespread or an isolated phenotype. A nucleotide alignment of the entire 9-kb region revealed it is present in over 83 sequenced bacterial genomes with >80% nucleotide identity and >70% query coverage, all of which are gammaproteobacteria. With the exception of 1 Shewanella and 2 Serratia genomes, the rest are Enterobacteriales: 33 Klebsiella, 29 Enterobacter, 11 Citrobacter, 3 Escherichia, 2 Raoultella, a Phytobacter, and 1 Metakosakonia. Based on these findings, with our stringent alignment parameters, the 9-kb region may not be widely present in all isolates of a particular Enterobacteriales species but is widely present in many species. As an example, Enterobacter cloacae clinical isolate Mu208 was observed to encode the same 9-kb region as R/S. We found that the 9-kb region in Mu208 also duplicated in the presence of colistin (see Fig. S6A in the supplemental material), contributed to survival on nickel (see Fig. S6B), and increased ncrABC-dependent nickel resistance when grown in the presence of colistin (see Fig. S6B). These data suggest that colistin-induced, ncrABC-dependent nickel resistance is likely a broadly relevant phenomenon.

In addition, further bioinformatic analyses allowed us to make insights into the regulation of the ncrABC operon. NcrA is a predicted MFS family protein (PFam PF07690) and NcrC is a predicted transmembrane protein which has amino acid analogy to the Ni and Co efflux proteins NirC (PFam PF03824) (17, 18) and RcnA (19) in Enterobacteriales. NcrB is a predicted helix-turn-helix transcriptional regulator with
structural homology to RcnR and CsoR, which are part of a family of metal-sensing negative regulators of metal efflux systems in other organisms (PFam PF02583) (20). NcrB contains 9 histidine residues (out of 87 amino acids), and this amino acid is known to bind nickel, suggesting that NcrB could be a regulator of ncrABC. We first showed that growth of wild-type (WT) R/S in the presence of nickel resulted in a marked increase in the expression level of ncrC (see Fig. S7A). Consistent with NcrB functioning as a negative regulator of the operon, deletion of ncrB led to a significant increase in expression of ncrC (see Fig. S7B). In addition to the expression data, the ncrB deletion strain exhibited a robust increase in nickel resistance while ncrA or ncrC mutants had a decrease in nickel resistance (see Fig. S7C). It is interesting that the ncrA and ncrC mutants have modest but complementable phenotypes (see Fig. S7D and E) relative to the ncrABC deletion strain, suggesting that they may have some overlapping function that is apparent only when both are deleted. The enhanced nickel resistance of ΔncrB correlated with decreased intracellular levels of this metal relative to WT R/S as measured by ICP-AES (Thermo iCAP 7400) (see Fig. S7F). Taken together, these data demonstrate that colistin selects for a nickel-resistant subpopulation of R/S cells harboring an amplification of a nickel resistance locus and highlight that antibiotic treatment can promote increased metal resistance (see Fig. S8).

Amplification of antibiotic resistance genes has recently been demonstrated to occur in some examples of heteroresistance (21, 22). This leads to a subpopulation of cells with higher antibiotic resistance gene dosage and increased resistance. In the present study of a colistin-heteroresistant isolate, we observe a gene amplification that is selected by the antibiotic (colistin) but which is not a determinant of the heteroresistance phenotype. Therefore, it is important to note that the observation of a gene amplification upon treatment with a specific stress (i.e., antibiotic) should not automatically be interpreted as indicating that the amplified gene(s) mediate the heteroresistance phenotype.

Multiple studies have found that bacterial exposure to metals can confer resistance to antibiotics and that metal and antibiotic resistance genes are often encoded on the same mobile genetic elements (23–26). In contrast, the effect of antibiotics on bacterial metal resistance has been unclear. The present study shows that antibiotic treatment can lead to metal resistance since colistin led to enhanced nickel resistance via duplication of ncrABC. These findings highlight an important consequence of antibiotic use, warning that these drugs could prime bacterial populations for survival on bactericidal metal-coated surfaces and thus enhance colonization of the hospital environment, leading to subsequent transmission and infection (see Fig. S8).

Data availability. All data are provided in the manuscript, in the supplemental materials, or are available upon request from the authors. The primer and nucleotide list (see Table S2 in the supplemental material) includes sequences and cloning information referenced in the manuscript. The DNA sequencing data have been deposited at NCBI under BioProject no. PRJNA263343.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.6 MB.
FIG S2, TIF file, 0.5 MB.
FIG S3, TIF file, 0.1 MB.
FIG S4, TIF file, 0.1 MB.
FIG S5, TIF file, 0.1 MB.
FIG S6, TIF file, 0.1 MB.
FIG S7, TIF file, 0.4 MB.
FIG S8, TIF file, 0.3 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, DOCX file, 0.02 MB.
ACKNOWLEDGMENTS

We thank the members of the Weiss lab, especially Jessie Wozniak and Siddharth Jaggavarapu, for helpful discussions about the manuscript. ICP-AES was performed and analyzed by Penn State University LIME Academic Research Service. D.A.H. and J.E.C. receive support from a postdoctoral research fellowship from the Cystic Fibrosis Foundation. D.S.W. is supported by NIH grant AI141883 and the Burroughs Wellcome Fund Investigator in the Pathogenesis of Infectious Disease award. C. L. is supported by 1K23HL138461-01A1.

REFERENCES

1. O’Neill J. 2016. Tackling drug-resistant infections globally: final report and recommendations. Review on Antimicrobial Resistance, London, United Kingdom. https://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf.

2. Burnham JP, Olsen MA, Kollef MH. 2019. Re-estimating annual deaths due to multidrug-resistant organism infections. Infect Control Hosp Epidemiol 40:112–113. https://doi.org/10.1017/ice.2018.304.

3. van Duin D, Kaye KS, Neuner EA, Bonomo RA. 2013. Carbapenem-resistant Enterobacteriaceae: a review of treatment and outcomes. Diagn Microbiol Infect Dis 75:115–120. https://doi.org/10.1016/j.diagmicrobio.2012.11.009.

4. White AP, Gibson DL, Kim W, Kay WW, Surette MG. 2006. Thin aggregative pilus formation of Escherichia coli O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. Int J Food Microbiol 156:133–140. https://doi.org/10.1016/j.ijfoodmicro.2012.03.014.

5. Oliveira M, Vinas I, Usall J, Anguera M, Abadías M. 2012. Presence and survival of Escherichia coli O157:H7 on lettuce leaves and in soil treated with contaminated compost and irrigation water. Int J Food Microbiol 156:133–140. https://doi.org/10.1016/j.ijfoodmicro.2012.03.014.

6. Weingarten RA, Johnson RC, Conlan S, Ramsburg AM, Dekker JP, Lau AF, Kilp P, Odom RT, Deming C, Park M, Thomas PJ, Henderson DK, Palmore TN, Segre JA, Frank KM. 2018. Genomic analysis of hospital plumbing biofilms reveals diverse reservoir of bacterial plasmids conferring carbapenem resistance. mBio 9:e02011-17. https://doi.org/10.1128/mBio.02011-17.

7. Guh AY, Bulens SN, Mu Y, Jacob JT, Reno J, Scott J, Wilson LE, Vaeth E, Jaggavarapu, for helpful discussions about the manuscript.

8. Querido MM, Aguilar L, Neves P, Pereira CC, Teixeira JP. 2019. Self-disinfecting surfaces and infection control. Colloids Surf B Biointerfaces 178:1–21. https://doi.org/10.1016/j.colsurfb.2019.02.009.

9. Villapun VM, Dover LG, Cross A, Gonzalez S. 2016. Antibacterial metallic touch surfaces. Materials (Basel) 9:736. https://doi.org/10.3390/ma9090736.

10. Napier BA, Band V, Burd EM, Weiss DS. 2014. Colistin heteroresistance in Acinetobacter baumannii. Nat Microbiol 1:6053. https://doi.org/10.1038/nmicrobiol.2016.53.

11. Band VI, Crispell EA, Napier BA, Herrera CM, Tharp GK, Vavikolanu K, Pohl J, Read TD, Bosinger SE, Trent MS, Burd EM, Weiss DS. 2016. Antibiotic failure mediated by a resistant subsatellite in Enterobacter cloacae. Nat Microbiol 1:6053. https://doi.org/10.1038/nmicrobiol.2016.53.

12. El-Halfawy OM, Valvano MA. 2015. Antibacterial heteroresistance: an emerging field in need of clarity. Clin Microbiol Rev 28:191–207. https://doi.org/10.1128/CMR.00058-14.

13. Partridge SR, Kwong SM, Firth N, Jensen SO. 2018. Mobile genetic elements associated with antimicrobial resistance. Clin Microbiol Rev 31:e00088-17.

14. Haritha A, Sagar KP, Tiwari A, Kimmanayi P, Rodrigue A, Mohan PM, Singh SS. 2009. MdrH, a novel metal resistance determinant of Pseudomonas putida KT2440, is flanked by metal-inducible mobile genetic elements. J Bacteriol 191:5976–5987. https://doi.org/10.1128/JB.00465-09.

15. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645. https://doi.org/10.1073/pnas.10163297.

16. Band VI, Hufnagel DA, Jaggavarapu S, Sherman EK, Wozniak JE, Satola SW, Farley MM, Jacob JT, Burd EM, Weiss DS. 2019. Antibiotic combinations that exploit heteroresistance to multiple drugs effectively control infection. Nat Microbiol 4:1627–1635. https://doi.org/10.1038/s41564-019-0480-z.

17. Tian J, Wu N, Li J, Liu Y, Guo J, Yao B, Fan Y. 2007. Nickel-resistant determinant from Leptospirillum ferriphilum. Appl Environ Microbiol 73:2364–2368. https://doi.org/10.1128/AEM.00207-07.

18. Park JS, Lee SJ, Rhie HG, Lee HS. 2008. Characterization of a chromosomal nickel resistance determinant from Klebsiella oxytoca CCUG 15788. J Microbiol Biotechnol 18:1040–1043.

19. Rodrigue A, EFFANTIN G, Mandrand-Berthelot MA. 2005. Identification of rccA (yohH), a nickel and cobalt resistance gene in Escherichia coli. J Bacteriol 187:2912–2916. https://doi.org/10.1128/JB.187.8.2912-2916.2005.

20. Higgins KA, Giedroc D. 2014. Insights into protein allosteroy in the CsoR/Crrn family of transcriptional repressors. Chem Lett 43:20–25. https://doi.org/10.1246/cl.130965.

21. Nicoloff H, Hjort K, Levin BR, Andersson DL. 2019. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. Nat Microbiol 4:504–514. https://doi.org/10.1038/s41564-018-0342-0.

22. Anderson SE, Sherman EX, Weiss DS, Rather PN. 2018. Aminoglycoside heteroresistance in Acinetobacter baumannii AB5075. mSphere 3:e00271-18. https://doi.org/10.1128/mSphere.00271-18.

23. Pal C, Asiani K, Arya S, Rensing C, Stekel DJ, Larsson DGI, Hobman JL. 2017. Metal resistance and its association with antibiotic resistance. Adv Microb Physiol 70:261–313. https://doi.org/10.1017/amspps.2017.20011.

24. Cavaco LM, Hasman H, Stegger M, Andersen PS, Skov R, Fluit AC, Itø T, Aarestrup FM. 2010. Cloning and occurrence of ccrC, a gene conferring cadmium and zinc resistance in methicillin-resistant Staphylococcus aureus CC398 isolates. Antimicrob Agents Chemother 54:3605–3608. https://doi.org/10.1128/AAC.00584-10.

25. Hözel CS, Muller C, Harms KS, Mikolajewski S, Schafer S, Schweiger K, Bauer J. 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. Environ Res 113:21–27. https://doi.org/10.1016/j.envres.2012.01.002.

26. Hu HW, Wang JT, Li J, Shi XZ, Ma YB, Chen D, He JZ. 2017. Long-term nickel contamination increases the occurrence of antibiotic resistance genes in agricultural soils. Environ Sci Technol 51:790–800. https://doi.org/10.1021/acs.est.6b03383.