Title

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Journal

Environmental Science and Technology, 54(14)

ISSN

0013-936X

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Publication Date

2020-07-21

DOI

10.1021/acs.est.0c01155

Environmental Science & Technology

pubs.acs.org/est

Article

Exposure to Environmental Levels of Pesticides Stimulates and Diversifies Evolution in *Escherichia coli* toward Higher Antibiotic Resistance

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Cite This: https://dx.doi.org/10.1021/acs.est.0c01155

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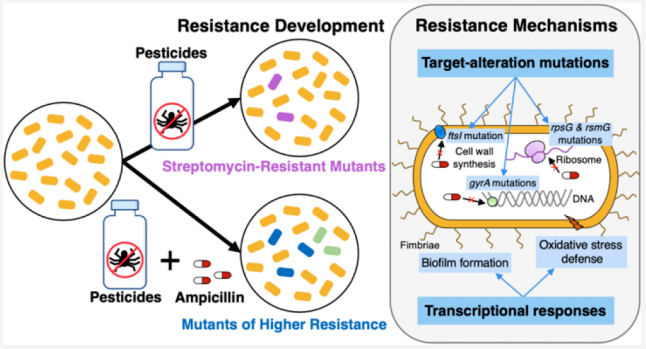
ABSTRACT: Antibiotic resistance is one of the most challenging issues in public health. Antibiotics have been increasingly used not only for humans and animals but also for crop protection as pesticides. Thus, antibiotics often coexist with pesticides in some environments. To investigate the effects of the co-occurring, nonantibiotic pesticides on the development of antibiotic resistance, we conducted long-term exposure experiments using an *Escherichia coli* K-12 model strain. The results reveal that (1) the exposure to pesticides (in mg/L) alone led to the emergence of mutants with significantly higher resistance to streptomycin; (2) the exposure to pesticides (in μg/L) together with a subinhibitory level (in high μg/L) of ampicillin synergistically stimulated the selection of ampicillin resistance and the cross-resistance to other antibiotics (i.e., ciprofloxacin, chloramphenicol, and tetracycline). Distinct and diversified genetic mutations emerged in the resistant mutants selected from the coexposure to both pesticides and ampicillin. The genetic mutations likely caused a holistic transcriptional regulation (e.g., biofilm formation, oxidative stress defense) when grown under antibiotic stress and led to increased antibiotic resistance. Together, these findings provide important fundamental insights into the development of antibiotic resistance and the resistance mechanisms under environmentally relevant conditions where antibiotics and nonantibiotic micropollutants coexist.



INTRODUCTION

Antibiotic resistance has become one of the most challenging environmental and public health issues. The *de novo* mutation is one important route for bacteria to acquire antibiotic resistance, under both clinical and environmental conditions.[1−4] Antibiotics at both inhibitory [i.e., above the minimal inhibitory concentration (MIC); typically, in the mg/L range] and subinhibitory concentrations (i.e., below the MIC; in the ng/L to high μg/L range) can lead to the emergence of increased resistance.[1,3] This may explain the emergence of antibiotic resistance in several nonclinically relevant environments, such as domestic sewage, water bodies receiving treated sewage from wastewater treatment plants, as well as farm runoffs, where antibiotics occur from tens of ng/L to a few hundreds of μg/L.[5−7]

Meanwhile, in natural and built environments, a variety of other emerging organic contaminants such as pesticides, nonantibiotic drugs, and personal care products are usually co-occurring with antibiotics at low levels (ng/L−μg/L each).[8−10] It is still unclear how these nonantibiotic contaminants would affect the selection of antibiotic resistance. If these contaminants and antibiotics have synergistic effects, especially at environmentally relevant concentrations, the development of antibiotic resistance in those environments would be overlooked or underestimated. Therefore, it is crucial to have a better understanding of the emergence of antibiotic resistance under long-term exposure to both antibiotics and nonantibiotic organic contaminants at environmental levels.

Among the contaminants coexisting with antibiotics, pesticides are one important group. They are typically found in agricultural soils, run-offs, and the receiving water bodies,[11−13] which are also potentially antibiotic-impacted environments. For example, antibiotics used in farms to treat sick animals and boost livestock growth can be released into the agricultural environments, leading to the co-occurrence of pesticides and antibiotics.[14,15] Moreover, antibiotics can also be applied to fight against bacterial diseases in plant farms, where pesticides are widely used. For instance, two antibiotics, streptomycin and oxytetracycline, are heavily used to combat citrus greening, a bacterial infection killing orange trees.[16] In addition, pesticides are found in antibiotic-impacted environ-

Received: February 24, 2020
Revised: May 22, 2020
Accepted: June 17, 2020
Published: June 17, 2020

ments such as irrigation water and municipal wastewater.[17−21] The environmental levels of individual pesticide range from less than one ng/L to tens of μg/L,[11,22] which brings an overall occurrence level to a high μg/L range due to the presence of many pesticide species used for different purposes in farms and households. Although many pesticides may not have a negative effect on bacterial growth, certain pesticides have similar inhibitory mechanisms to some antibiotics, such as membrane disruption (e.g., biocides, such as chlorhexidine and fenticlor) and inhibition of cell wall synthesis (e.g., herbicide dimethyl tetrachloroterephthalate, DCPA).[23−27] Thus, exposure to those pesticides might also affect the selection of antibiotic resistance. Short-term exposure studies using very high levels of herbicides (hundreds of mg/L to g/L) revealed changes (increase or decrease) in antibiotic susceptibility and accelerated evolution of resistance.[28,29] However, it is still unknown whether long-term exposure to pesticides at more environmentally relevant levels could favor evolution toward higher antibiotic resistance.

The goal of this study was to fill the knowledge gap regarding the emergence of antibiotic resistance in environments with the occurrence of both antibiotics and pesticides. We aimed to investigate the effects of environmental-level pesticides alone and their interactive effects (synergistic, antagonistic, or neutral) with sub-MIC antibiotics on the development of antibiotic resistance and then identify the underlying mechanisms. To accomplish our goals, we designed evolutionary experiments with a susceptible *Escherichia coli* strain exposed to pesticides only and coexposed to ampicillin (Amp) and pesticides at environmentally relevant concentrations for 500 generations. The change in antibiotic resistance of *de novo* mutants was determined. Genetic mutations developed from different exposure conditions were identified by whole-genome sequencing (WGS). Transcriptional responses to antibiotics in the resistant mutants from the coexposure and the Amp-exposure were examined using RNA sequencing (RNA-seq) and quantitative reverse transcription qPCR (RT-qPCR). Stimulative effects of pesticide exposure on the development of antibiotic resistance were observed. The distinct mechanisms of antibiotic resistance developed under the co- and Amp-exposure were proposed and discussed.

MATERIALS AND METHODS

Bacterial Strains, Growth, and Selection Conditions. The antibiotic-susceptible bacterium used in this study was *Escherichia coli* K-12 C3000 (*E. coli*). Luria-Bertani (LB) medium was used in all exposure experiments. The detailed cultivation method can be found in the "Supplemental Methods" section of the Supporting Information (SI). The pesticide mixture used for exposure experiments consisted of 23 frequently detected pesticides in various natural and engineered environments (Table S1).[21,30−33] The concentrations of pesticides used in this study were based on their environmental concentrations (1EC) (0.1−4.8 μg/L, each; ∼20 μg/L in total) reported in the literature (Table S1). Two exposure scenarios were investigated (Figure S1): (1) Exposure to the pesticide mixture only, with a series of concentrations at 1/125EC, 1/25EC, 1/5EC, 1EC, 5EC, 25EC, 125EC. This was taking into account the variation of exposure levels in different environments where degradation or accumulation of pesticides could occur. *E. coli* grown on LB medium only was set up as the control. (2) Coexposure to pesticides at 1EC and Amp at 1/125MIC_0, 1/25MIC_0, or 1/5MIC_0 (MIC_0, the minimum inhibitory concentration of antibiotics for the ancestor *E. coli* strain, G0, in LB medium. $MIC_{0,\ Amp}$ = 4 mg/L). The corresponding controls included the exposure to ampicillin only and the exposure to pesticides only. We used ampicillin as the representative antibiotic co-occurring with pesticides because it is widely used in agriculture and animal husbandry.[34] The exposure experiments for each condition were conducted in 96-well plates with a total culture volume of 200 μL. The cell cultures were serially transferred (1:500 dilution) into another 96-well plate (containing fresh LB medium with the same concentrations of Amp and/or pesticides) every 24 h (∼9 generations per passage). (See Supplemental Methods in the SI.) The cultures were incubated at 30 °C and aerated by shaking. By the serial passage, the cultures were exposed to the designated exposure conditions for a long-term period, i.e., 500 generations (∼56 days of passage). All the exposure conditions were performed with three *E. coli* populations in parallel (biological triplicates). The MIC of each population was determined after 500 generations. Populations that showed an increase of MIC by more than 1.5-fold compared to MIC_0 were subjected to further investigation on the MIC distribution of isolated resistant mutants. An overall layout of the experimental setup was illustrated in Figure S1 of the SI.

Isolation of Resistant Mutants and Determination of Minimum Inhibitory Concentrations. The MIC_0 of the ancestor strain (G0) was determined by MIC tests applied to 5 different types of antibiotics: Amp, tetracycline (Tet), ciprofloxacin (Cip), streptomycin (Strep), and chloramphenicol (Chl) following standardized protocols of MIC tests (see Supplemental Methods in SI).[35] Briefly, the overnight cell culture was diluted to a cell density of OD_{600} = 0.1 and inoculated into fresh LB medium containing antibiotics at a series of concentrations. These tested cell cultures were incubated at 35 °C for 20 h, and OD_{600} was measured. The MIC was determined as the lowest concentration that can totally inhibit cell growth (i.e., < 0.1× OD_{600} of cell culture without antibiotics).[36]

For the isolation of resistant mutants, after 500-generation exposure, 5× diluted cell cultures were spread on LB agar plates containing individual antibiotics at 1× MIC_0 (i.e., 4 mg/L Amp, 1 mg/L Tet, 0.016 mg/L Cip, 9 mg/L Strep, and 8 mg/L Chl). Colonies grown on those selective plates were considered as antibiotic resistant mutants. Twelve resistant mutants were randomly picked up on the selective agar plate inoculated with each of the three parallel populations, resulting in a total of 36 resistant mutants for each exposure condition. The MICs of these resistant mutants were further determined (see Supplemental Methods in the SI). The Mann-Whitney U test was performed to analyze the difference of MICs of resistant mutants under different exposure conditions (p-value <0.05, N = 36).

DNA Extraction, WGS, and SNP Calling. Susceptible isolates from the evolved populations without chemical exposure, Strep-resistant (Strep-R) mutants from the evolved populations under pesticide exposure, Amp-resistant (Amp-R) and Cip-resistant (Cip-R) mutants from the evolved populations under the coexposure and the Amp-exposure were selected and cultivated overnight. On the basis of the MIC values, the top three out of the 36 resistant mutants isolated under each exposure condition were selected for the whole genome sequencing. Cell pellets were collected by centrifugation. The genomic DNA was extracted using DNeasy

https://dx.doi.org/10.1021/acs.est.0c01155
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Blood and Tissue Kits (Qiagen) according to the manufacturer's instructions and then subjected to Illumina MiSeq 250-bp paired-end sequencing. A dynamic sequence trimming was done by SolexaQA software[37] with a minimum quality score of 24 and a minimum sequence length of 50 bp. The trimmed reads of G0 were aligned against the *E. coli* K-12 MG1655 genome (NC_000913.3) using Bowtie 2 toolkit[38] to assemble the G0 genome. All reads from isolated mutants were then aligned against the assembled G0 genome. SAMtools[39] and Picard Tools[40] were used to format and reformat the intermediate-alignment files. SNPs and INDELs were identified using the Genome Analysis Toolkit UnifiedGenotyper,[41] with the calling criteria of >5-read coverage and >50% mutation frequency.[42] Valid genetic mutations were identified as the ones that lead to amino acid sequence changes and are not in the susceptible isolates from the G500 populations without chemical exposure. Relative abundances of selected SNPs in populations under different exposure conditions were determined using the customized rhAmp SNP genotyping assay (see Supplemental Methods in the SI).

RNA Extraction, RNA-Seq, and Differential Gene Expression Analyses. One Amp-R mutant and one Cip-R mutant were selected among the sequenced mutants from each exposure condition. These mutants were grown to an OD_{600} of 0.75 in 8 mL LB broth in a shaking incubator at 35 °C for 5 h. Three biological replicates were performed for each selected mutant. The cultures were then treated with the respective antibiotic (i.e., Amp for the Amp-R mutant and Cip for the Cip-R mutant) at 0.8× MIC. Cultures were then allowed to grow for 30 min, and the cell pellets were collected by centrifugation.

Total RNA was isolated according to the acid phenol:chloroform extraction method[43] and treated with DNase to remove residual DNA using TURBO DNA-free kit (Thermo Fisher Scientific). Ribosomal RNA was removed, and sample libraries of resistant mutants with antibiotic treatment were built using the TruSeq mRNA-Seq Library Preparation Kit (Illumina, USA) according to the manufacturer's recommendations. Sequencing was performed on a HiSeq 2500 system and produced 100-bp single-end reads. Low-quality reads (quality score <30, sequence length <25 bp) were removed using SolexaQA software.[37] The qualified sequences were subject to the alignment using the Bowtie 2 toolkit[38] against the assembled genome of G0. Genes were counted using FeatureCounts software,[44] and the count data were then analyzed using R version 3.5.1 and Bioconductor package DESeq2 version 3.8.[45] Genes were considered to have significantly differential expressions based on three criteria: (i) TPM (transcripts per million) > 5 in at least one sample; (ii) FDR adjusted *p*-value <0.05; (iii) > 2-fold difference in TPM values between the two exposure conditions. The RNA-seq results were then validated by RT-qPCR (see the Supplemental Methods in the SI) using specifically designed primer sets (Table S2).

RESULTS AND DISCUSSION

Effects of the Exposure to Pesticides on the Development of Streptomycin Resistance. According to the MICs of *E. coli* populations exposed to pesticides after 500 generations, the populations exposed to 125EC pesticides showed a 1.5-fold increase in MIC of streptomycin ($MIC_{500,\ Strep}$ = 1.5 $MIC_{0,\ Strep}$). We further investigated the MIC distribution of the 36 resistant mutants randomly picked up from the populations exposed to 125EC pesticides and the control populations with no exposure. Their MICs were compared to the MIC of the ancestor strain (MIC_0). Mutants from the 125EC pesticide exposure acquired significantly higher Strep MICs compared to the mutants from the no exposure control (*p*-value = 0.0013, *N* = 36) (Figure 1),

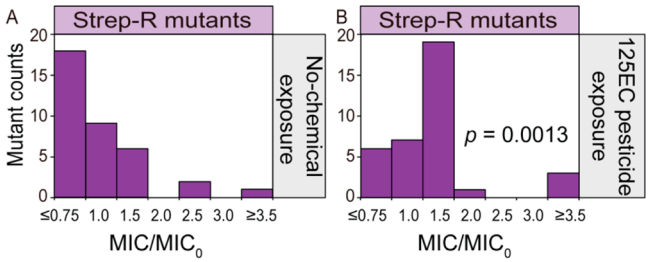

Figure 1. MICs of mutants from the no exposure control (A) and mutants from high-level (125EC) pesticide exposure (B) after 500 generations. $MIC_{0,\ Strep}$ is the MIC (9 mg/L) of the ancestor strain (G0). The *p*-value of the Mann−Whitney U test is indicated (*N* = 36).

indicating that exposure to pesticides alone at a level as high as 125EC stimulated the emergence of stronger streptomycin resistance. It is worth noting that the exposure to 125EC pesticides only selected *de novo* mutants more resistant to Strep, but not to the other four tested antibiotics (i.e., Amp, Tet, Cip, and Chl). In addition, *E. coli* populations exposed to pesticides concentrations lower than 125EC (i.e., 1/125, 1/25, 1/5, 1, 5, and 25EC) did not acquire higher MICs of all tested antibiotics compared to the no exposure control, which showed the same resistance as the ancestor strain G0 (MIC = MIC_0).

Effects of the Coexposure to Pesticides and Amp on the Development of Antibiotic Resistance. Among the three coexposure conditions to Amp and pesticides (i.e., (1/125MIC_0, 1EC), (1/25MIC_0, 1EC), and (1/5MIC_0, 1EC)), the exposure to 1/5MIC_0 Amp and 1EC pesticides exhibited a statistically significant increase in growth compared to the Amp-exposure control (1/5 MIC_0, 0) when grown in LB medium containing 1× MIC_0. Thus, *E. coli* populations under this exposure condition were subject to the isolation of resistant mutants from the populations and investigation of MIC distributions of the isolated resistant mutants. Results show that the 36 Amp-R mutants from the coexposure (i.e., 1/5MIC_0 Amp and 1EC pesticides) exhibited a shift to higher MICs (Figure 2B), compared to those from the exposure to 1/5MIC_0 Amp only (Figure 2A). The shift of MICs was statistically significant, according to the Mann-Whitney U test (*p*-value = 0.039). As no increase in resistance was observed by exposing the cells to 1EC pesticides only, the higher resistance developed after the coexposure compared to the Amp-exposure was due to the synergistic effect of pesticides when a subinhibitory level of antibiotic selection pressure was also present. To explore the development of cross-resistance, we determined the MICs of the mutants from coexposed and Amp-exposed populations (G500) resistant to four other antibiotics: Strep, Chl, Cip, and Tet. Except for similar Strep resistance (i.e., MICs) of mutants from the two exposure conditions, significantly higher (1.5−3.5 times) resistance to the other three antibiotics was observed in the mutants from the coexposed populations than from the Amp-exposed populations (*p*-values of 1.1×10^{-10}, 0.044, and 1.3×10^{-7}

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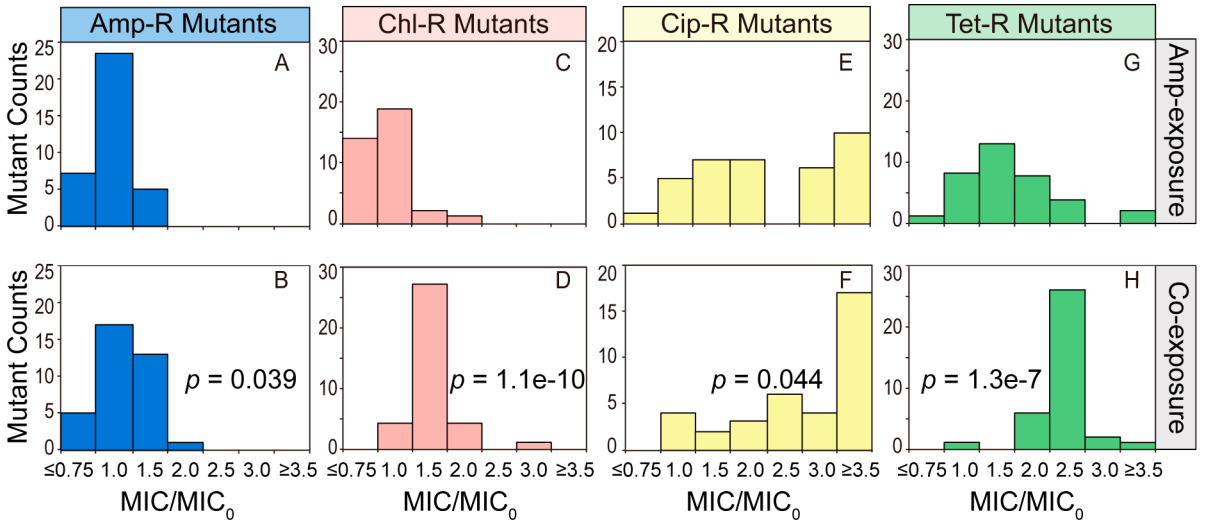

Figure 2. MICs of resistant mutants from the Amp-exposure ($1/5MIC_{0,\ Amp}$) (A, C, E, G) and the coexposure (1EC pesticides $+1/5MIC_{0,\ Amp}$) (B, D, F, H). $MIC_{0,\ Amp}$ = 4 mg/L, $MIC_{0,\ Chl}$ = 8 mg/L, $MIC_{0,\ Cip}$ = 0.016 mg/L, and $MIC_{0,\ Tet}$ = 1 mg/L. The *p*-values of the Mann−Whitney U tests between the Amp-exposure and the coexposure are indicated (N = 36).

for Chl, Cip, and Tet, respectively) (Figure 2 D,F,H). Thus, besides the acquirement of stronger resistance to Amp, the coexposure also synergistically led to the development of stronger cross-resistance to other antibiotics that the cells had not been exposed to.

Genetic Mutations in Strep-Resistant Mutants from the Pesticide Exposure. The genomes of Strep-R mutants isolated from G500 populations exposed to 125EC pesticides revealed four valid genetic mutations, including two SNPs and two deletions when compared to the genomes of the susceptible isolates from G500 *E. coli* without chemical exposure (Figure 3, see Table S3 for a complete list of genetic mutations). These four mutated genes encode proteins for (i) target modification (i.e., *rsmG* and *rpsG*) and (ii) regulation (i.e., *arcB* and *sspA*). The *rsmG* mutation is known to cause streptomycin resistance in *E. coli* and other bacterial strains according to prior studies.[46,47] All three sequenced Strep-R mutants shared the same genetic mutation in the *rpsG* gene (SNP: A → T), resulting in gaining a stop codon (*) replacing Leu157 in the amino acid sequence (Table S3). The *rpsG* gene encodes the subunit S7 of 30S ribosomal protein, which is part of the target protein of streptomycin. The higher resistance of mutants from the pesticide exposure could be attributed to this target-modified mutation. Notably, this specific mutation in *rpsG* has not been reported in Strep-induced mutants before and thus might be specifically induced under the pesticide exposure condition.

Genetic Mutations in Resistant Mutants Selected from the Coexposure. We identified distinct mutations in Amp-R mutants from the coexposure compared to those from the Amp-exposure. For all three sequenced Amp-R mutants from the coexposed cultures, the same mutation occurred in the *ftsI* gene (SNP: A → T; amino acid change: Gln536 → Leu) (Figure 3). It encodes an Amp-binding protein, and this genetic mutation likely altered the protein structure, hence lowering the affinity of Amp to its target protein.[48] Such mutation on *ftsI* might result in the resistance to other beta-lactam antibiotics, which act by binding to FtsI. Notably, at the population level, the same genetic mutation was only present in the resistant populations (selected by 1× MIC_0) from the coexposed cultures compared to the cultures exposed to Amp

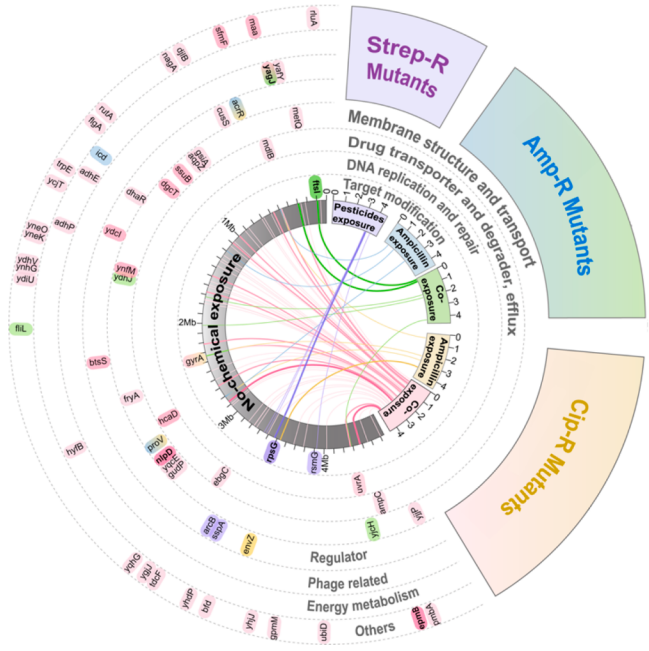

Figure 3. Genetic mutations identified in the sequenced resistant mutants. The links represent valid genetic mutations. The capsules stand for mutated genes and their positions on the genome. The darker the colors and the thicker the links, the higher are the frequencies of the genetic mutations detected among the three resistant mutants. Rings with dashed borders contain the genes involved in a specific function.

only, according to the results of SNP genotyping assays (Figure S2). It further demonstrates that the *ftsI* mutation was specifically induced under the coexposure. In addition, multiple mutations occurred in a prophage-related gene *yagJ*. Mutations also occurred in genes encoding membrane and flagellar structure proteins (Table S3). The structural alteration of these proteins could potentially limit or avoid the entry of the antibiotic into the cells, thus resulting in increased resistance. In contrast, fewer valid genetic mutations (three mutated genes: *acrR*, *proV*, and *icd*) were detected in the Amp-

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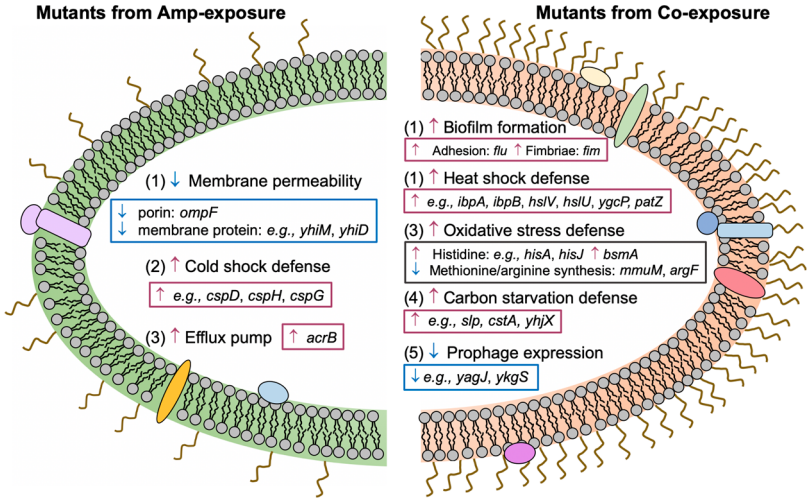

Figure 4. Proposed transcriptional-level mechanisms in response to antibiotic stress shared in Amp- and Cip-resistant mutants from the Amp- and coexposure conditions (up and down arrows represent up- and down-regulated gene expression between the Amp- and coexposure conditions).

R mutants from the Amp-exposure, none of which was shared with the resistant mutants from the coexposure (Figure 3). The mutations in *acrR* have been known to be associated with multidrug resistance.[48,49] The *acrR* gene encodes the repressor regulating the expression of *acrAB* genes, which encode a multidrug efflux pump in *E. coli*. The mutation of this gene might repress its expression, which in turn would increase the expression of *acrAB* and activate the efflux to increase resistance.[49] Collectively, the distinct genetic mutations selected from the coexposure compared to those from the Amp-exposure suggest different resistance mechanisms, which may contribute to the higher Amp resistance developed from the coexposure.

For Cip-R mutants isolated from the coexposed populations, the *gyrA* mutations occurred in all three sequenced mutants: two had Ser83 → Leu, and one had Asp87 → Gly (Table S3). The DNA gyrase encoded by *gyrA* is the target of Cip, and the mutations in *gyrA* could lead to Cip resistance.[48] Along with *gyrA* mutations, more diverse genetic mutations were detected in Cip-R mutants from the coexposure compared to the Amp-exposure (Figure 3, Table S3). The mutated genes are involved in various functions (Table S3): (i) DNA replication and repair, (ii) drug transporter, degrader, and efflux pumps, (iii) membrane structure and transporter, (iv) regulator, (v) prophage, and (vi) energy metabolism. Most of those mutations were not known to cause Cip resistance directly. Nevertheless, the coexposure seemed to stimulate the evolution by diversifying the mutation sites, thus resulting in a higher chance of selecting mutants with higher Cip resistance. In comparison, there were only four genetic mutations in the Cip-R mutants from the Amp-exposure, which occurred in genes encoding proteins for target modification, transporters, and regulators (Figure 3, Table S3). Except for the mutation in *gyrA* (SNP: Asp87 → Gly) that was also found in one mutant from the coexposure condition, all the other three mutations only occurred in the three Cip-R mutants selected from the Amp-exposed culture. Two mutants had a mutation (SNP: T → C, Thr120 → Ala) in the *envZ* gene that encodes a membrane-associated protein kinase, which might reduce the production of membrane porin and lead to antibiotic resistance.[50] The same genetic mutation in the efflux pump regulator gene *acrR* in the Amp-R mutants was found in one of the three Cip-R mutants from the Amp-exposed culture (Figure 3, Table S3), again suggesting that a more general resistance mechanism that can cause cross-resistance, such as extrusion of antibiotics by efflux pump, was employed by resistant mutants developed under the Amp-exposure.

Transcriptional Responses of Resistant Mutants from the Coexposure and the Amp-Exposure. To further investigate the mechanisms of higher antibiotic resistance developed under the coexposure compared to the Amp-exposure, differential gene expression analysis at the transcriptional level was conducted using RNA-seq. Principal component analysis using normalized transcript abundances indicates clear differences between resistant mutants from the coexposure and those from the Amp-exposure when grown under antibiotic stress (0.8× MIC) (Figure S3A,B). A total of 91 and 107 genes exhibited significantly higher/lower expression in Amp-R mutants and Cip-R mutants from the coexposure, respectively, compared to those from the Amp-exposure (Figure S4). The expression results of representative genes were validated using RT-qPCR (Figure S5). We further grouped the differentially expressed genes into eight categories according to their functions. We inferred the differences in resistance mechanisms at the transcriptional level between the resistant mutants from the Amp- and the coexposure conditions.

For the Amp-R mutant from the Amp-exposure, the expression of many genes encoding membrane proteins (e.g., *yhiD*, *yhiM*) was less expressed compared to that of the coexposure condition (Figure 4, Figure S4A). The decreased expression of these genes may decrease the cell membrane permeability, inhibiting the entry of antibiotics. The gene involved in lysine decarboxylation showed higher expression in the mutant selected from the Amp-exposure (Figure S4A). An increase in lysine decarboxylase (CadA) activity could lead to cadaverine accumulation, which can result in the reduction of membrane permeability,[51] hence increasing the tolerance to antibiotics. Meanwhile, several genes involved in cold shock defense (e.g., *cspHG*) showed significantly higher expression than those from the coexposure (Figure 4, Figure S4A). The bacterial adaption to cold shock was found to increase the resistance to antibiotics and other stresses.[52,53] In contrast, the

https://dx.doi.org/10.1021/acs.est.0c01155
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Amp-R mutant from the coexposure exhibited higher expression of genes involved in fimbriae structure (*fimBE*), cell attachment (*flu*), heat shock defense (*ibpAB* and *hslVU*), carbon starvation defense (*slp*), and acid stress defense (*hde*) (Figure 4, Figure S4A). In addition, the histidine synthesis genes were upregulated in the mutant from the coexposure. Histidine may produce antioxidants in response to oxidative stress,[54] thus helping with antibiotic stress defense. Meanwhile, many genes involved in central metabolic activities, including arginine synthesis (e.g., *argF*), methionine synthesis (*mmuM*), galactose transport (*mglA* and *mglB*), ferric iron reduction (*fhuF*), and fatty acid *β*-oxidation (*fadB*), exhibited lower expression (Figure S4A). The downregulation of these genes was reported to be associated with oxidative stress defense.[55−59] Prophage genes (e.g., *yagJ*, *ykgS*) were completely deactivated (Figure S4A). It has not been well understood how the function loss of prophage genes would affect the antibiotic resistance, and opposite effects were observed from a limited number of studies, where the deletion of prophages in bacterial genomes improved[60,61] or reduced[62] the tolerance to antibiotics and other environmental stresses.

The genes differentially expressed in the Cip-R mutants from the Amp-exposure and coexposure conditions suggested similar resistance mechanisms at the transcriptional level with the Amp-R mutants. For the Cip-R mutant from the Amp-exposure, the *ompF* gene, which is known to reduce membrane permeability and lead to antibiotic resistance,[63] showed lower expression. Additionally, higher gene expression was observed in the cold-shock defense gene *cspD* and the efflux gene *acrB* (Figure 4, Figure S4B). As a comparison, the Cip-R mutant from the coexposure exhibited higher expression of genes related to fimbriae structure (*fim*), heat shock defense (*patZ* and *ygcP*), oxidative stress defense (*bsm*A), and carbon starvation defense (*cstA* and *yhjX*) (Figure 4, Figure S4B), which were consistent with the types of genes differentially expressed in the Amp-R mutant from the coexposure. The genes involved in *N*-acetylneuraminate degradation (e.g., *nanA*) also exhibited higher expression in the Cip-R mutant from the coexposure (Figure S4B), which may facilitate biofilm formation.[64] The expression of genes involved in methionine synthesis (*mmuM*) and prophage genes (i.e., *yagJ*, *ykgS*) was completely shut down (Figure S4B), which was the same as in the Amp-R mutant from the coexposure, suggesting a general transcriptional response leading to higher resistance to multiple antibiotics. Additionally, *yagJ* was the mutated gene detected in both Amp-R and Cip-R mutants from the coexposure, the deactivation of which was also observed when grown without antibiotic stress (Figure S6). It indicates that such differential gene expression was independent of antibiotic treatment and was due to the mutation of *yagJ* (Figure S6). Similarly, the deactivation of *yagJ* and/or the other distinct genetic mutations in the resistant mutants from the coexposure may cause the differential expression independent of antibiotic stress observed for the other genes, including *fimB* that controls fimbriae formation (Figure S6).

Together, the difference in transcriptional responses of resistant mutants from the coexposure and the Amp-exposure is likely due to the distinct genetic mutations developed under the two exposure conditions. In addition to resistance caused directly by the genetic mutations, the transcriptional responses of mutants from the Amp-exposure that may contribute to the resistance phenotype include (i) reduced membrane permeability, (ii) cold shock defense, and (iii) induction of efflux pumps. Whereas, the resistant mutants from the coexposure may gain higher resistance via (i) increased biofilm formation, (ii) heat shock defense, (iii) oxidative stress defense, (iv) carbon starvation defense, and (v) deactivation of prophage-related genes (Figure 4). Those general transcriptional-level resistance mechanisms triggered by the exposure to one antibiotic may also lead to resistance to other coexisting stresses, such as multiple drug resistance.

Environmental Implications. This study reports a previously overlooked yet critical phenomenon regarding the development of antibiotic resistance. That is, long-term exposure to environmental levels of pesticides alone and together with sub-MIC levels of antibiotics can stimulate and diversify *de novo* mutations toward higher resistance to certain antibiotics. The pesticide level (mg/L), which triggered evolution toward higher resistance to Strep, may occur in agricultural soils, biosolids, and aquatic organisms where pesticides can be accumulated.[22,65] Moreover, various levels (ng−*μ*g/L) of pesticides can co-occur with sub-MIC level antibiotics[16] at agricultural sites where both pesticides and antibiotics may be applied, in the after-application run-offs as well as in aquatic environments receiving the surface runoff and WWTP effluents.[8−10] Such co-occurrence may synergistically select for *de novo* antibiotic-resistant mutants from a susceptible population, with higher resistance than those that could have been selected by antibiotic alone. If that was the case, an observed increase in antibiotic resistance in certain environments could be attributed to the presence of not only antibiotics but also some nonantibiotic micropollutants such as pesticides. Future exposure studies may be needed to determine whether there are specific pesticides among the 23 tested ones that can exhibit the synergistic effect. In this study, Amp, which has been widely used and detected in agriculture and animal husbandry,[34] was used in the exposure experiments as the representative antibiotic co-occurring with pesticides. Future studies can be conducted to investigate whether similar synergistic effects of pesticides could be observed when coexposed to other beta-lactams and other types of antibiotics. The coexposure is more likely to stimulate mutations in the genes encoding antibiotic-targeted proteins, thus resulting in higher resistance. Mutations in nonantibiotic-resistance genes (e.g., the prophage-related gene *yagJ*) may also play a role in the acquisition of higher antibiotic resistance from the coexposure, suggesting the need for additional indicative biomarkers and further demonstration. In addition, the different transcriptional responses in resistant mutants from the coexposure could be caused by distinct genetic mutations and contribute to the higher resistance phenotype. These findings provide new insights into selection pressures and mechanisms of antibiotic resistance under more environmentally relevant exposure conditions as well as helpful information to assess potential environmental impacts during certain agricultural operations such as antibiotic application and the reuse of biosolids and treated wastewater.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c01155.

(PDF)

https://dx.doi.org/10.1021/acs.est.0c01155
Environ. Sci. Technol. XXXX, XXX, XXX−XXX

Accession Codes

All WGS and RNA sequencing data have been deposited in the NCBI SRA database under accession no. PRJNA530028.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to give thanks to Hernandez Alvaro Gonzalo and Chris L. Wright at the Roy J. Carver Biotechnology Center, University of Illinois at Urbana−Champaign for whole-genome sequencing and RNA sequencing support.

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