Site-specific target-modification mutations exclusively induced by the coexposure to low levels of pesticides and streptomycin caused strong streptomycin resistance in clinically relevant *Escherichia coli*

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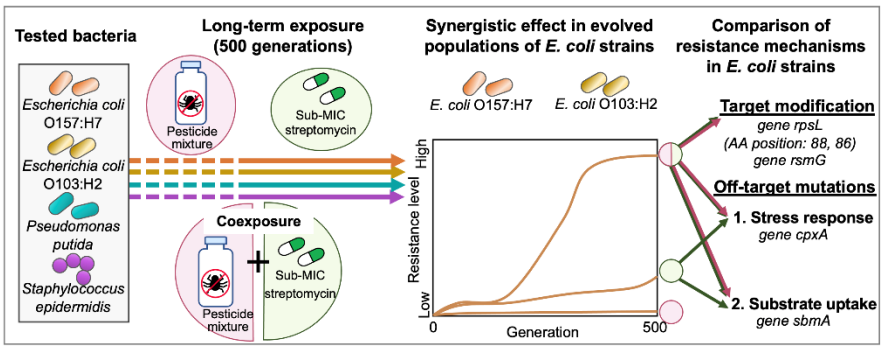
Abstract

Environmentally relevant levels of pesticides may promote the emergence of stronger streptomycin-resistant mutants in an *Escherichia coli* K-12 strain in the presence of sublethal levels of streptomycin. However, it is not clear whether this synergistic effect exists in other strains within and outside the *Escherichia* genus. Here, we investigated the long-term evolution toward stronger antibiotic resistance under the pesticides and streptomycin coexposure in bacterial strains of three different genera, including pathogenic *E. coli* strains O157:H7 and O103:H2, one *Pseudomonas* strain, and one *Staphylococcus* strain. Consistently, the coexposure induced significantly stronger streptomycin-resistant mutants in the two *E. coli* strains. However, it did not promote any evolution toward stronger streptomycin resistance in the *Pseudomonas* and *Staphylococcus* strains. Site-specific mutations of genes, such as *rpsL*(Lys88Arg), which encode streptomycin target proteins were exclusively evolved in the coexposed *E. coli* strains and conferred 80-fold increase in streptomycin resistance. These findings imply that a higher risk of strong and inheritable streptomycin resistance of *E. coli* strains, including the pathogenic ones, may exist in certain environments where pesticides and antibiotics cooccur.

Keywords

Antibiotic resistance; Pesticides; Streptomycin; Selective pressure; *Escherichia coli*; Evolution

Graphical abstract



1. Introduction

Given the global threat to public health caused by antibiotic resistance (O'Neill, 2016), successful combat against antibiotic resistance requires a holistic and multisectoral approach to reduce the rate at which antibiotic-resistant bacteria evolve and spread in humans, animals, and the environments (Vikesland et al., 2017). While exposure to antibiotics may select for antibiotic-resistant bacteria, among the environmental compartments, other selective pressures that could promote the microbial evolution to antibiotic resistance are not well determined. A comprehensive understanding of selective pressures in the environment is essential to accurately assess the risks associated with antibiotic resistance evolution, thus facilitating the identification of appropriate mitigation strategies.

Some studies report that nonantibiotic selective pressures, such as heavy metals, disinfectants, disinfection byproducts, certain pharmaceuticals, and herbicides, could induce antibiotic resistance in *Escherichia coli* strains (a few to thousands mg/L, ppm) (Jin et al., 2018; Kurenbach et al., 2015; Li et al., 2016; Li et al., 2019; Lu et al., 2018). Some nonantibiotic-induced changes in antibiotic resistance were observed in other bacterial strains (Kurenbach et al., 2015; Lv et al., 2014) or microbial communities (Kim et al., 2018). Furthermore, our recent study reveals that the coexposure to a pesticide mixture at environmental levels and sublethal levels of antibiotics (ampicillin or streptomycin) synergistically promoted the long-term antibiotic resistance evolution in the *Escherichia coli* K-12 strains (Xing et al., 2021; Xing et al., 2020). The coexposure to pesticides and streptomycin is of greater concern since the latter is not only applied in clinics but also in agriculture to treat bacterial diseases (McKenna, 2019; Vidaver, 2002). Thus, in the related environments where both antibiotics and pesticides are occurring, the synergistic

44 effect in promoting antibiotic resistance might be overlooked, and the risks of antibiotic resistance
45 development could be underestimated.

46 Different bacteria, e.g., with/without pathogenesis and from different genera, may have
47 different responses to the exposed environments. Thus, identifying the influenced bacteria is very
48 important for the risk assessment of nonantibiotic contaminants as selective pressures. So far, it is
49 yet unclear whether the synergy of pesticides and antibiotics in the evolution of antibiotic
50 resistance would be the same for pathogenic *E. coli* strains, as well as bacterial strains from
51 different genera. To fill the knowledge gap, we conducted evolutionary experiments under the
52 coexposure conditions with environmental levels of pesticides and streptomycin (below minimal
53 inhibitory concentrations, MIC) in species from different genera, including two pathogenic *E. coli*
54 O157:H7 and O103:H2, one *Pseudomonas* species (*P. putida*), and one *Staphylococcus* species (*S.*
55 *epidermidis*) (Rasmussen and Casey, 2001). The change in antibiotic resistance levels of the
56 evolved populations was determined after 500 generations. We then identified genetic mutations
57 shared in the populations with stronger streptomycin resistance developed. We demonstrated
58 different resistance phenotypes of mutants carrying the commonly emerged genetic mutations
59 identified in the evolved populations.

60

61 **2. Materials and methods**

62 *2.1 Bacterial strains, growth, selection conditions, and evolutionary experiments*

63 The bacterial strains used in this study were purchased from ATCC: The Global
64 Bioresource Center, including an *E. coli* O157:H7 strain (ATCC No. 43888), an *E. coli* O103:H2
65 strain (which was kindly received from Dr. Abasiofiok Mark Ibekwe from the Salinity Laboratory
66 of USDA in Riverside), one *P. putida* strain (ATCC No. 12633), and one *S. epidermidis* strain

(ATCC No. 14990). The *sbmA* knockout *E. coli* strain (JW ID: JW0368) and its parent strain (BW25113) were obtained from Horizon Discovery Company. The growth media for all the bacterial strains was Luria-Bertani (LB) broth, and liquid cultures were aerated by shaking. First, the stock cells for each strain were revived, and then a single colony was picked up from the streaked LB agar plates of the revived culture, which was regarded as the ancestor strain. All ancestor strains (i.e., *E. coli* O157:H7, *E. coli* O103:H2, *P. putida*, and *S. epidermidis*) were susceptible to streptomycin with the initial MIC of 7, 8, 4, 8 mg/L, respectively, which were used for the following evolutionary experiments.

The selection conditions included exposures to streptomycin (Strep) alone or a mixture of 23 pesticides, which were frequently detected in aquatic environments (Supplementary Table S1) (Xing et al., 2021; Xing et al., 2020), as well as the coexposure to Strep and pesticides. The pesticides included eight herbicides (e.g., phenylureas and chlorotriazines), seven insecticides (i.e., carbaryl, carbofuran, diazinon, fipronil, imidacloprid, chlorpyrifos, and metaldehyde), six fungicides (e.g., benzimidazoles and triazole fungicides), one biocide (i.e., irgarol), and one commonly used insect repellent (i.e., DEET). The selection concentration of Strep was at sub-MIC level (i.e., 1/5 MIC_0, MIC_0 is the Strep MIC of the ancestor strain) [denoted (1/5,0)]. For each pesticide, we selected a representative environmental concentration (EC) based on previous reports (Supplementary Table S1) (0.1 – 4.8 μg/L each and ~ 20 μg/L in total). We applied three pesticide exposure levels, which were 1, 10, and 100 times of EC, corresponding to the occurrence levels of pesticides at various environmental exposure scenarios [denoted (0,1), (0,10), (0,100), respectively]. The coexposure conditions thus were combinations of 1/5 MIC_0 and different concentrations of pesticides [denoted (1/5,1), (1/5,10), (1/5,100), respectively]. Control

experiments in the absence of selective pressures (Strep or pesticides) were also set up (Supplementary Fig. S1).

Evolutionary experiments were performed as described previously (Xing et al., 2020). Briefly, we serially transferred eight replicate lineages for 500 generations in 200 μL LB liquid media under one exposure condition in a 96-well plate. Multiple exposure conditions were included on the same plate. The pesticide mixture was prepared in methanol, added to the wells, and evaporated prior to adding LB media and the Strep stock solution (freshly made in MilliQ water). The cell cultures were incubated at 30 °C in a 150-rpm shaker in the dark for 24 hours, diluted 500 folds, and inoculated into fresh LB media containing the same selective chemicals. Each transfer resulted in log2(500) = ~ 9 generations, and the evolutionary experiments lasted for 56 days. The experimental setup and workflow were also depicted in Supplementary Fig. S1. The cultures after every 100 generations were preserved by adding 100 μL of 50% glycerol and stored at −80 ºC.

2.2 MIC test of evolved populations

Every 100 generations, the evolved populations were subject to MIC tests, which determine phenotypic resistance levels of the populations. The cell culture was diluted with 0.9% NaCl solution to an OD_{600} of 0.1, which was regarded as the "standard solution". Then 0.5 μL of the standard solution was added into fresh LB medium containing Strep with a series of concentrations. In the growth control, 0.5 μL of the standard solution was added to fresh LB medium plus 5 μL of nanopore water instead of the antibiotic solution. The negative control was the same as growth control but without the inoculum. Cell cultures were incubated at 30 °C for 20 hours, and then the OD_{600} was measured. The MIC was determined as the concentration that completely inhibited cell growth based on the OD_{600} measurement. We then performed the Student's t-test to analyze the

112 significance of MIC differences between the coexposure conditions and single exposure (p-value
113 < 0.05, $N = 8$, unpaired, two-tailed, unequal variances).
114 *2.3 DNA extraction and whole-population sequencing*
115 To identify the antibiotic resistance mechanisms in the evolved populations of *E. coli*
116 O157:H7 and *E. coli* O103:H2 from different exposure conditions, after 500 generations, we
117 sequenced selected populations under different exposure conditions, which have developed
118 increased levels of antibiotic resistance. The evolved populations without chemical exposure were
119 also sequenced to identify genetic adaptations to the growth conditions. Each evolved population
120 was cultivated overnight in LB medium, and cell pellets were collected by centrifugation. Genomic
121 DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (Qiagen), and the gDNA
122 concentrations were determined on a Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington,
123 DE). The gDNA was then subjected to 150-bp paired-end sequencing on the Illumina NextSeq
124 platform, which was carried out by Microbial Genome Sequencing Center. The mutant alleles were
125 called out by the workflow described previously (Xing et al., 2021; Xing et al., 2020). A dynamic
126 sequence trimming was done by SolexaQA software (Cox et al., 2010) with a minimum quality
127 score of 30 and a minimum sequence length of 50 bp. All samples were aligned against the *E. coli*
128 O157:H7 ATCC 43888 genome and *E. coli* O103:H2 genome available at NCBI GenBank
129 (NZ_CP041623.1 and AP010958.1) using the Bowtie 2 toolkit (Langmead and Salzberg, 2012).
130 SAMtools was used to format and reformat the intermediate-alignment files (Li et al., 2009). SNPs
131 and INDELs were identified and annotated with software BCFtools (Li, 2011) and SnpEff
132 (Cingolani et al., 2012). Among these, the valid mutant alleles were further filtered based on the
133 criteria: (i) causing amino-acid-sequence change, (ii) not found in the ancestor G_0 and the evolved
134 populations without selective pressures at generation 500, (iii) > 20-read coverage, and (iv) > 5%

(1/20) mutant allele frequency at the mutation positions, indicating the specific genotype in the populations with larger than 5% presence.

2.4 Isolation of resistant mutants, SNP genotyping assays, and whole-genome sequencing

To determine the correlation between *rpsL* mutations at different amino acid positions and their phenotypic resistance levels, we isolated resistant mutants from the evolved populations of *E. coli* O157:H7. The cell culture was spread on selective LB agar plates with 1× MIC_0 Strep and incubated overnight. The resistant clones were picked up, and three of them were confirmed to be *rpsL*-mutation-positive via the SNP genotyping assays. The SNP genotyping assays we applied in this study were Custom TaqMan SNP Genotyping Assays (Thermo Fisher Scientific). Two assays were designed specifically targeting the *rpsL* (Leu49Gln) mutation and the *rpsL* (Lys88Arg) mutation. The assays were performed in 96-well plates on a real-time PCR instrument QuantStudio 3 (Thermo Fisher Scientific) according to the manufacturer's instructions and recommended thermal cycling conditions. The "Genotyping" application in Thermo Fisher Cloud was used to analyze the mutant genotype. Eight *rpsL* mutants were subjected to whole-genome sequencing to obtain a comprehensive list of mutations in the *rpsL* mutants. The SNP calling procedures were the same as the analysis of whole-population sequencing data described above, except that the mutation frequency cutoff was set to 50%.

3. Results and discussion

3.1 Synergistic effects of pesticides and streptomycin on the selection of antibiotic resistance in bacterial populations of different genera

For *E. coli* O157:H7 (Fig. 1A), all of the three coexposure levels stimulated strong resistance development (> 40-fold increase in MIC). It is much stronger than the resistance

158 developed in populations exposed to Strep alone (mild to moderate resistance, i.e., 4 – 10 folds
159 increase in MIC; p-value = 0.04, 0.01, and 2×10^{-5} between the coexposure and the Strep-only
160 exposure, for 1, 10, and 100EC, respectively according to the Student's t-test). As the added
161 pesticide concentrations increased from 1EC to 100EC, more replicate lineages (i.e., from 4/8 to
162 6/8) have evolved the high-level resistance (Fig. 1A). The exposure to pesticides alone did not
163 significantly increase the Strep MIC of the evolved *E. coli* O157:H7 populations. We observed a
164 similar trend in *E. coli* O103:H2 (Fig. 1B). The coexposure led to strong antibiotic resistance (i.e.,
165 20 – 50 folds, 2 to 3 out of 8 replicate lineages), albeit with no statistical significance according to
166 the Student's t-test. In comparison, the exposure to Strep alone was not able to increase Strep
167 resistance by more than 5 folds. The synergistic effect of Strep and pesticides on Strep resistance
168 development in *E. coli* O157:H7 and *E. coli* O103:H2 is consistent with what has been observed
169 in another *E. coli* strain (K-12) with the same exposure levels (Xing et al., 2021). It suggests that
170 the impact of Strep and pesticide coexposure on the selection of stronger Strep resistance may
171 occur in a broader spectrum of *E. coli* strains. Differently, for *P. putida* and *S. epidermidis*, no
172 synergistic effect of Strep and pesticide coexposure was observed. For *P. putida*, the Strep-only
173 exposure caused the emergence of mild Strep resistance (4 – 6-fold increase in MIC) (Fig. 1C).
174 However, the additional exposure to pesticides did not select for stronger Strep resistance than the
175 exposure to Strep alone. None of the exposure conditions showed an impact on Strep resistance in
176 the *S. epidermidis* populations after 500 generations, which remained similar to the original level
177 (Fig. 1D). These results suggest that the synergistic effect of Strep and pesticides on antibiotic
178 resistance development is likely to be specific to certain bacteria, such as *E. coli*.

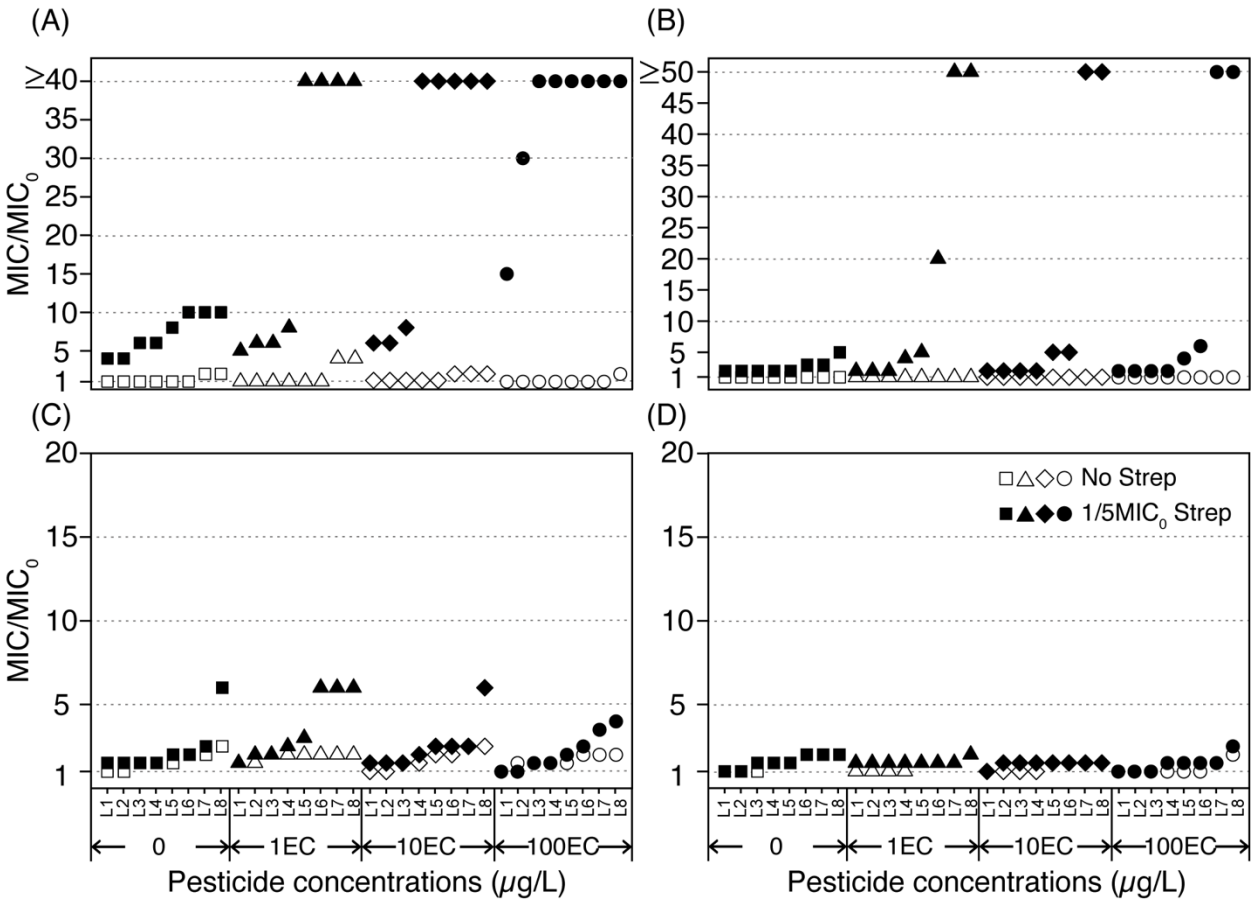


Fig. 1. Population MICs of *E. coli* O157:H7 (A), *E. coli* O103:H2 (B), *P. putida* (C), *S. epidermidis* (D) under exposures to Strep alone, pesticides alone, and Strep+pesticides after 500-generation evolution (L1-L8: the eight replicate lineages; The MICs of the *E. coli* O157:H7, *E. coli* O103:H2, *P. putida*, and *S. epidermidis* ancestor strains are 7, 8, 4, and 8 mg/L, respectively).

We further examined the Strep resistance trajectories of the evolved *E. coli* O157:H7 populations during 500 generations. Most of the replicate lineages from the sub-MIC Strep selection alone have gradually evolved with increased resistance but did not develop strong resistance (< 10 folds) after 500 generations (Fig. 2A). In contrast, four populations from (1/5,1) condition exhibited > 40-fold increase in resistance (Fig. 2B). The strong resistance was acquired at generation 500. Compared to 1EC, 10EC and higher could accelerate the emergence of stronger

Strep resistance. Moreover, as the pesticide concentration increased, more lineages acquired stronger Strep resistance (> 10-fold increase) after 500 generations (4, 5, and 8 for 1EC, 10EC, and 100EC, respectively) (Fig. 2C&D). It indicates a dose-effect of pesticides as the co-stressor on synergistically inducing and accelerating the evolution toward stronger resistance.

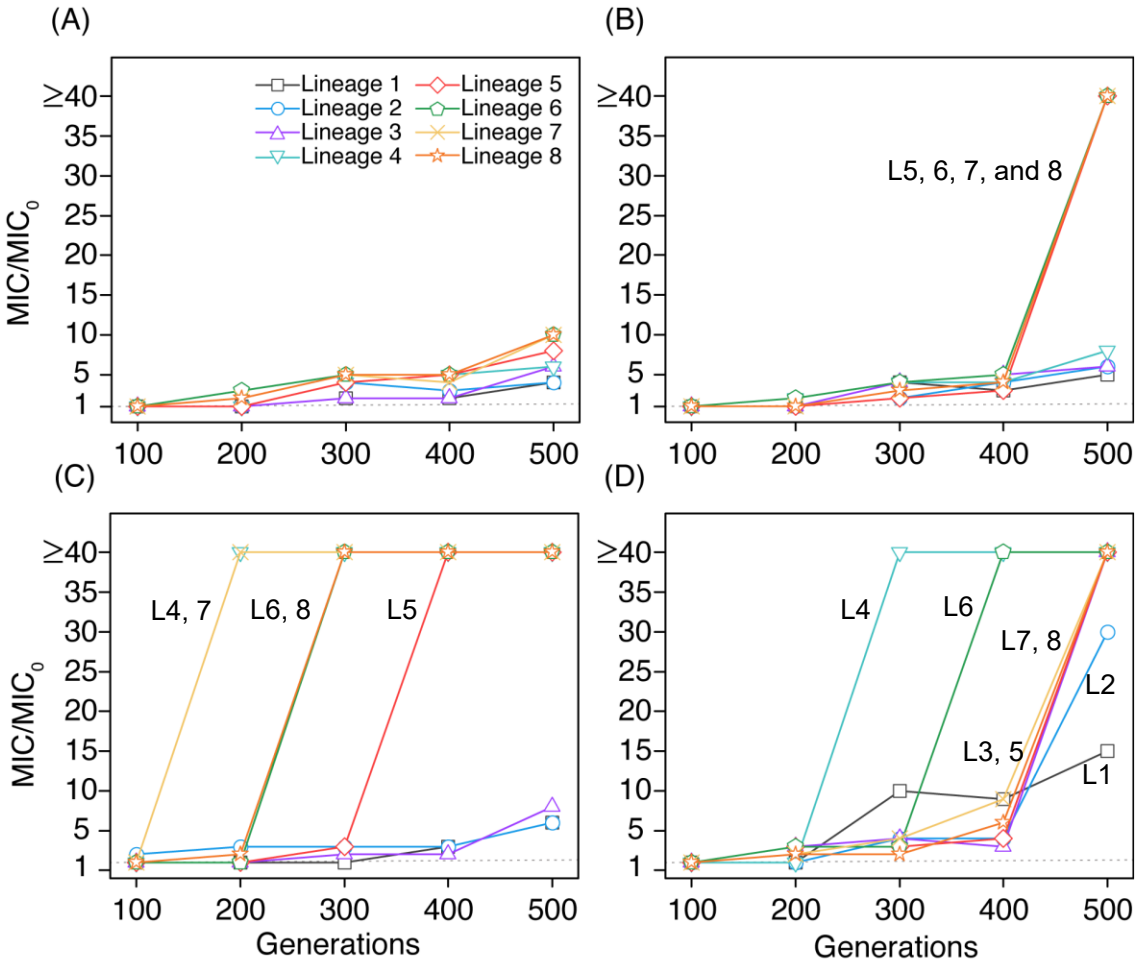


Fig. 2. Trajectories of population MICs of *E. coli* O157:H7 exposed to Strep alone (A) and pesticides and Strep coexposure [B: (1/5,1); C: (1/5,10); D: (1/5,100)] over 500 generations (Note: lineages with substantial increase in Strep resistance were labeled next to the trajectory line).

3.2 Site-specific target-modification mutations in rpsL caused the high streptomycin resistance in coexposed E. coli populations

We identified mutations in the evolved populations of *E. coli* O157:H7 and *E. coli* O103:H2 at generation 500 from the coexposure and Strep-only exposure to compare the genetic basis of the developed Strep resistance. The resistance mechanisms of evolved *E. coli* O157:H7 and O103:H2 populations from the coexposure condition were mainly associated with mutations of genes involved in (i) target modification, (ii) DNA replication and transcription, (iii) stress response, (iv) substrate uptake, (v) fimbriae, flagella, and motility, (vi) phage (i.e., *FNZ21_RS17055* gene function), and (vii) metabolism (Figure 3). Among them, similar mutations in genes involved in stress response, substrate uptake, motility, phage, and metabolism were also detected in the evolved populations exposed to Strep alone (Figure 3).

The high-level Strep resistance in the coexposed populations was likely caused by the distinct mutations, which were not induced by Strep exposure alone. In *E. coli* O157:H7, the distinct mutations were mainly *rpsL* mutations (i.e., Lys88Arg, Lys88Thr, and Lys88Gln) (Fig. 3A). Gene *rpsL* encodes the Strep-target ribosomal S12 protein. Consistently, single-amino-acid-substitution mutations at position 88 (Lys88Arg and Lys88Gln) have also been reported to cause strong Strep resistance in multiple strains (Fukuda et al., 1999; Hosokawa et al., 2002; Oz et al., 2014; Westhoff et al., 2017). Other distinct mutations that developed under the coexposure and likely contributed to strong resistance were those in genes related to cell motility (e.g., *fliM*, *mot* genes) and phage proteins (i.e., *FNZ21_RS20200*) (Fig. 3A). Similarly, in *E. coli* O103:H2, distinct mutations in *rpsL* (i.e., Lys43Asn, Lys43Arg, Arg86Ser) were exclusively induced in the coexposed populations that showed a substantial increase in Srep resistance (Fig. 3B). The same *rpsL* mutation (Arg86Ser) was also identified in the highly resistant *E. coli* K-12 populations

exposed to the same pesticide co-stressors (Xing et al., 2021). In addition, mutations in mutator genes (i.e., *mutT* and *mutL*) were also identified in some of the coexposed *E. coli* O103:H2 (Fig. 3B), likely causing mutator phenotypes. The mutator phenotypes are known to create superior genetic backgrounds for selecting antibiotic resistance mutations (Chopra et al., 2003) or directly lead to high-level resistance (Couce et al., 2015). It can also explain the higher number of genetic mutations identified in the coexposed O103:H2 populations with *mutT* or *mutL* mutations than the O103:H2 without those mutations (Fig. 3B).

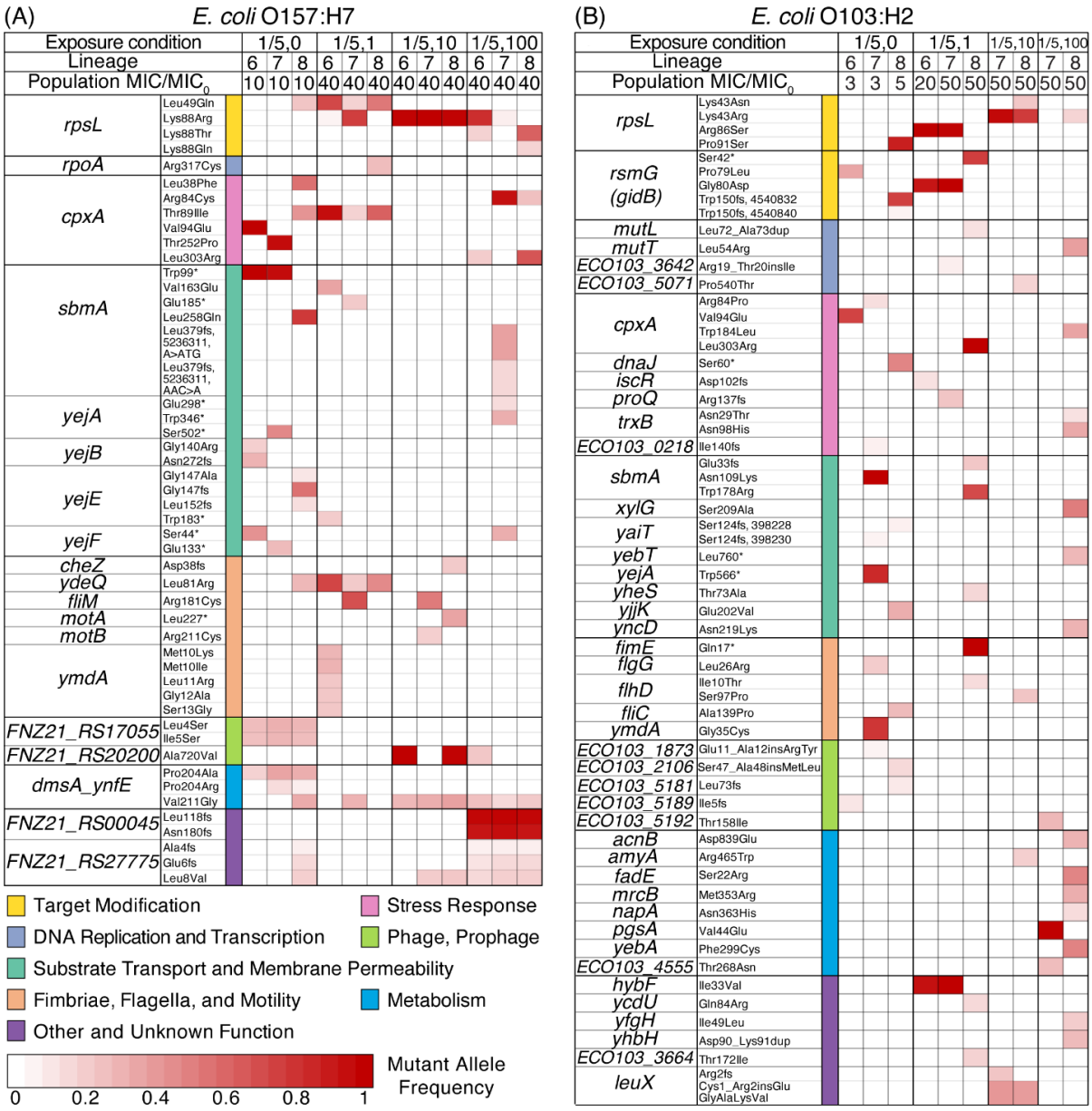


Fig. 3. Heatmap of the mutant allele frequency of mutations identified in the evolved populations with the coexposure and the Strep-alone exposure in *E. coli* O157:H7 (A) and *E. coli* O103:H2 (B).

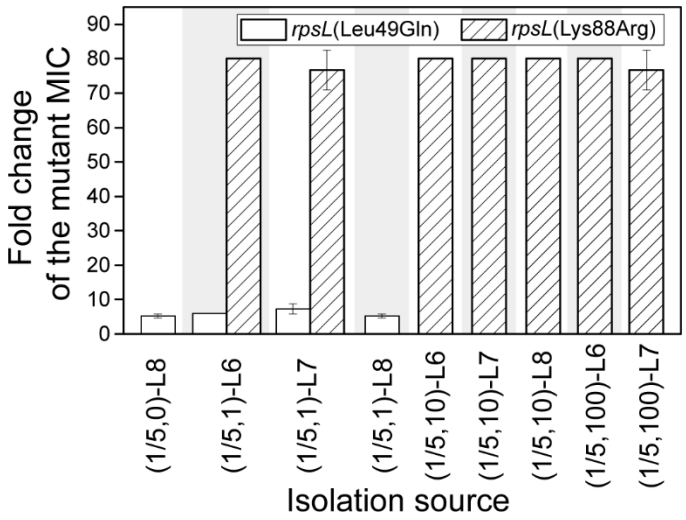


Fig. 4. The fold change of Strep MIC (MIC/MIC_0) for Leu49Gln and Lys88Arg *rpsL* mutants isolated from select lineages of *E. coli* O157:H7 under different exposure conditions [i.e., (1/5,0), (1/5,1), (1/5,10), and (1/5,100)].

Although target-modification mutations in gene *rpsL* were also identified in two evolved populations exposed to Strep alone (Fig. 3), they failed to cause strong Strep resistance. This led to the hypothesis that the target-modification mutations at different amino acid positions may lead to different levels of Strep resistance. To examine this, we isolated Strep-resistant mutants, which carry *rpsL* mutations at different amino acid positions, from different Strep-only and coexposed *E. coli* O157:H7 populations. While the *rpsL* (Leu49Gln) mutants from both (1/5, 0) and (1/5, 1) exposures only showed a 5 – 9-fold increase in Strep resistance compared to the ancestor strain, all the isolated *rpsL* (Leu88Arg) mutants from all coexposures, including the one mutant carrying only this *rpsL* mutation (Supplementary Table S3), exhibited a 70 – 80-fold increase (Fig. 4). One should note that the *rpsL* (Leu49Gln) mutants also carried mutations in 2 – 3 other genes, including *cpxA* and *sbmA* that are involved in stress response and substrate transport and may also contribute to resistance (Fig. 3, Supplementary Table S3). A combination of small-effect resistance mutations

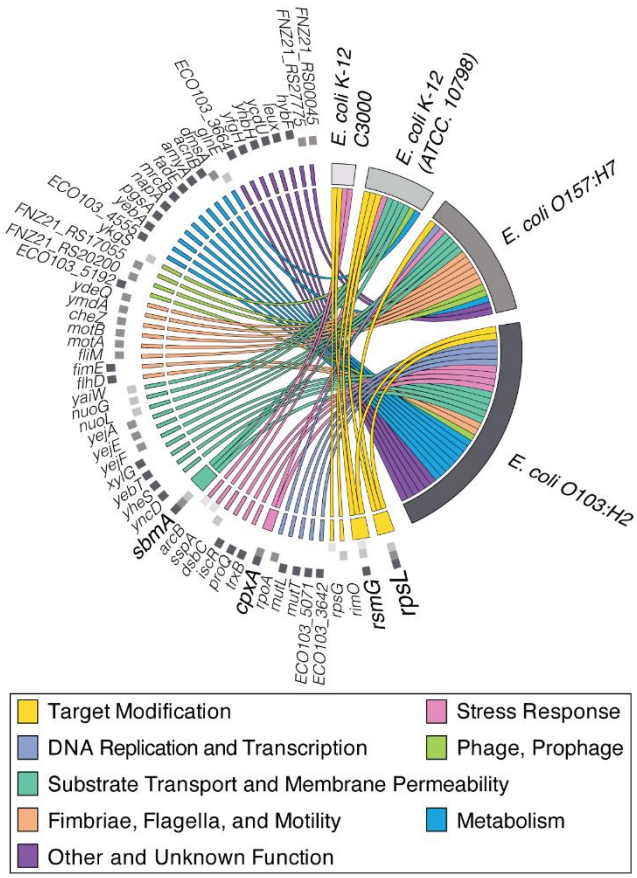
could lead to higher resistance (Wistrand-Yuen et al., 2018). Thus, *rpsL* (Leu49Gln) mutation alone would not lead to an increase in Strep resistance higher than what had been achieved in the *rpsL* (Leu49Gln) mutants with other genetic mutations. It indicates that *rpsL* mutations conferring strong Strep resistance are site-specific, and one confirmed amino acid position in *rpsL* was 88. Similarly, since *rpsL* mutations at amino acid positions 86 and 43 were dominant in some coexposed *E. coli* O103:H2 populations that showed strong Strep resistance (Fig. 3B), those two positions could also be specific *rpsL* mutation sites causing the strong resistance. Due to the cooccurrence of other genetic mutations, the exact role of the two mutation sites of *rpsL* needs to be further examined.

3.3 Commonly induced Strep resistance genetic mutations by the pesticide co-stressors in different E. coli strains

The effect of pesticide exposure on the development of Strep resistance has been observed in different *E. coli* strains, including the two pathogenic strains in this study and two K-12 derivatives (C3000 and ATCC 10798) in our previous studies (Xing et al., 2021; Xing et al., 2020). Thus, we compared the genetic mutations induced in those strains and identified the commonly shared ones that could indicate different Strep resistance levels (Fig. 5). The target-modification mutations, substrate-transport-related, and stress-response-related off-target mutations are the three types commonly developed in all investigated strains. Besides the above-demonstrated site-specific target-modification mutations in *rpsL* (i.e., Leu88Arg, Arg86Ser, and Lys43Arg), mutations in another Strep-target protein-encoding gene, *rsmG*, were also shared among the strains. This gene encodes a methyltransferase involved in the methylation of the 16S rRNA. The loss of RsmG activity could loosen the binding of Strep to the 30S subunit, leading to Strep resistance.

The isolated loss-of-function *rsmG* mutants of *E. coli* ATCC 10798 caused a 8 – 20-fold increase in Strep resistance (Xing et al., 2021).

Mutations in gene *sbmA* were highly shared off-target mutations, which were also induced by the Strep exposure alone. They emerged before 300 generations under the coexposure (Xing et al., 2021) and corresponded to a mild increase (3 – 10-fold) in Strep resistance. Mutations in gene *sbmA* included stop-gained mutations, frameshift mutations, and missense mutations (Fig. 3, Supplementary Table S2), which likely caused the loss of function or structural change of SbmA, an antimicrobial peptide transporter. A *sbmA*-knockout mutant of *E. coli* BW25113 exhibited a 4-fold increase in Strep resistance compared to the parent strain (Table S4), similar to our observations in the other *E. coli* with *sbmA* mutations. The resistance mechanism caused by *sbmA* mutations is still unclear, but mutations in this gene have also been identified in the previously reported bacteria resistant to aminoglycosides (Hoeksema et al., 2019; Jahn et al., 2017; Lázár et al., 2014).



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290 **Fig. 5.** Summary of identified mutations conferring streptomycin resistance in different *E. coli*
291 strains exposed to pesticides (i.e., *E. coli* K-12 C3000) or coexposed to streptomycin and pesticides
292 [i.e., *E. coli* K-12 (ATCC. 10798), *E. coli* O157:H7, and *E. coli* O103:H2].

293

294 Various stress-response-related genes were mutated in the four *E. coli* strains, and *cpxA*
295 mutations were found in the two pathogenic strains. The CpxA and CpxR system, which senses
296 and responds to periplasmic stress, has been implicated in antibiotic resistance (Batchelor et al.,
297 2005; Mahoney Tara and Silhavy Thomas, 2013). The *cpxA* mutations have been previously
298 identified from clinical samples and laboratory evolutionary experiments, which caused the
299 resistance to beta-lactams and aminoglycosides (Masi et al., 2020; Sun et al., 2009; Suzuki et al.,

2014). The *cpxA* mutations identified in this study were at different amino acid positions and likely conferred the same resistance spectrum but at a different level.

3.4 Environmental implications

The synergistic effect of pesticides and streptomycin in promoting the evolution toward stronger antibiotic resistance does not occur to all bacterial genera. Similar synergistic effects of environmental-level pesticides and low-level antibiotics in stimulating the emergence of stronger resistant mutants were observed in four *E. coli* strains, including the O157:H7 strain and the O103:H2 strain in this study and two others, *E. coli* K-12 C3000 (Xing et al., 2020) and *E. coli* K-12 ATCC. 10798 (Xing et al., 2021) (Fig. 4). Thus, the synergistic effect is likely specific to some bacteria, such as *E. coli*, which may survive and even grow in various open environments including soil and water (van Elsas et al., 2011). Moreover, *E. coli* O157:H7 and O103:H2 strains are of greater clinical relevance and have been recognized as important causes of diarrheal illness outbreaks (Galland et al., 2001; Luna-Gierke et al., 2014; Maal-Bared et al., 2013; Meng et al., 1998; Nüesch-Inderbinen et al., 2018; Solomakos et al., 2009), of which the resistant phenotypes are of greater concern. The increased resistance of these *E. coli* strains developed during the pesticides and streptomycin coexposure suggests even higher risks to public health if pathogenic *E. coli* were present in environments where pesticides and antibiotics could cooccur. Those environments include agricultural fields, surface water bodies receiving agricultural runoffs, and municipal wastewater treatment plants. Future studies using molecular biology and omics (genomic, transcriptomic, proteomic, and metabolomic) tools are needed to comprehensively examine the antibiotic resistance mechanisms of environmentally isolated *E. coli* strains, particularly the pathogenic ones.

Additionally, we demonstrated that site-specific target-modification mutations in *rpsL* were exclusively induced by the coexposure conditions and caused a substantial increase in Strep resistance. Furthermore, the specific strong-resistance mutation sites in *rpsL* we identified in *E. coli* strains, e.g., amino acid positions 43 and 88, also conferred strong Strep resistance in other genera (Fukuda et al., 1999; Hosokawa et al., 2002). The commonly occurring genetic mutations conferring antibiotic resistance could be signature genetic markers to evaluate antibiotic resistance levels. In complement with the detection of antibiotic resistance genes, which does not necessarily result in phenotypic resistance, the detection of site-specific mutations could be developed in the future for antibiotic resistance surveillance in various environments.

4. Conclusion

This study assessed the synergistic effects of the pesticide mixture and antibiotics on antibiotic resistance development in bacterial strains from different genera. The same effect of pesticides and Strep coexposure was observed in two pathogenic *E. coli* strains, but not *P. putida* and *S. epidermidis*. The development of stronger antibiotic resistance in pathogenic *E. coli* strains poses even higher risks to public health. Target-modification mutations (i.e., *rpsL* mutations) at specific sites exclusively emerged in several coexposed *E. coli* strains and caused high-level phenotypic resistance. Other off-target mutations commonly induced in coexposed *E. coli* populations were also identified. Those genetic mutations conferring antibiotic resistance could serve as additional biomarkers for a more accurate risk assessment of antibiotic resistance in the environment.

Supplementary material

346 The supplementary material includes Figure S1 and Table S1 – S4.

347

348 **Accession numbers**

349 All the sequencing data have been deposited in the NCBI SRA database under accession no.
350 PRJNA746605.

351 **Authors' contributing statement**

352 Y. M. and Y. X. conceived this study, D. H., S. Z., and Y. X. conducted the exposure experiments
353 and MIC tests. Y. X. conducted the sequencing analysis. X. K. and Y. X. performed mutant
354 isolation and SNP genotyping assays. All authors discussed the results. Y. X. and Y. M. wrote the
355 manuscript, and Y. M. supervised the projects.

356 **Acknowledgments**

357 This study was supported by the National Science Foundation (Award No. 2045658).

358 **Competing financial interests**

359 The authors declare no competing financial interest.

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