SYNERGISTIC EFFECT OF PESTICIDES AND STREPTOMYCIN ON THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN EVOLVED BACTERIAL STRAINS

By

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ABSTRACT

Antibiotics have been observed as a main selection pressure in different environments and even when detected at low levels, antibiotics have been found to stimulate de novo mutations resulting in resistant mutants. Nonetheless, the effects of non-antibiotic contaminants found in the environment and their contribution to the development of antibiotic resistance remains unknown. In recent studies we have found a link between environmental-level pesticides and streptomycin at low-levels resulting in a strong resistance from the evolved bacteria *Escherichia coli* in K-12 populations. Specific molecular mechanisms that resulted in phenotypic resistance were stimulated with pesticides. Although, this synergistic effect was already observed in the evolved bacteria *Escherichia coli* in K-12, it has not been observed or studied in other bacterial strains within the *Escherichia genus* or other bacterial genera. In this study, long-term evolutionary experiments were conducted with four different bacteria strains that included *E. coli* O157:H7, *E. coli* O103:H2, *P. putida*, and *S. epidermidis* who were exposed to environmental levels of pesticides and low-level streptomycin. The results from our study revealed that the synergistic effect of pesticides and streptomycin on increased levels of antibiotic resistance were more common in the *E. coli* strains exposed compared to the *P. putida*, and *S. epidermidis* strands tested in this study. In the *E. coli* O157:H7 and *E. coli* O103:H2 strains exposed to both pesticides and streptomycin, antibiotic resistance increased by approximately 40-50-fold. Coexposed bacterial populations that had an overall strong level of resistance, contained specific genetic mutations that encouraged phenotypic resistance occurred in target-modification mutations, motility-related mutations, and mutations within mutator genes. The results from this

study are critical to understanding and recognizing the dangers of antibiotic resistance in pathogenic *E. coli* where pesticides and antibiotics can coexist in the environment.

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Introduction

Antibiotics are fundamental in society because of their effectiveness in combating bacterial infections that would otherwise be lethal to human populations. Antibiotics are widely and typically utilized in hospitals, households, livestock, and agriculture among others.[1] This widespread use of antibiotics has become a cause for concern as antibiotic resistance in bacteria is becoming more frequently observed. Rapidly evolving bacteria groups have become invulnerable to antibiotics and are estimated to be the cause of death to nearly 23,000 individuals and has cost $55 billion each year to the United States.[1]

The most efficient technique to decrease the rate at which bacteria is becoming antibiotic resistant and spreading through humans, the environment, and animals requires the cooperation between different sectors in industry.[1] Antibiotics applied to agriculture are misused and carelessly added to prevent infection in crops but ultimately end up as runoff in environments where bacteria can become exposed and if suitable conditions permit, lead to antibiotic resistance.[2] In natural environments, antibiotics can be found in low concentrations that can range anywhere between ng/L to µg/L.[2] Antibiotics found in natural environments are susceptible to other potential selective pressures that could enhance the evolution of bacteria. Understanding and identifying the mechanisms through which contaminants and other selective pressures in the environment encourage the rate at which bacteria is evolving is crucial for future regulation of antibiotic use.

Previous studies have concluded that at high levels (> mg/L), selective pressures like heavy metals, disinfection byproducts, herbicide, and select pharmaceuticals have potential to increase antibiotic resistance in *Escherichia coli* strains.[2-6] A recent study conducted determined that *Escherichia coli* K-12 strains that had been coexposed to pesticides at levels similar to those

found in the environment (sub mg/L) and low-levels of the antibiotic's streptomycin and ampicillin, interdependently increased the bacteria's evolution potential leading to long-term resistance.[7,8] Streptomycin is one of the few antibiotics approved by the United States Environmental Protection Agency (EPA) for treating microbial disease in agriculture where there is a high likelihood to be exposed to pesticides.[9] Due to the constant interaction between streptomycin and pesticides in natural environments, the synergistic effect responsible for stimulating antibiotic resistance may be underestimated and requires extensive research.

To fill the knowledge gap, regarding whether multiple bacterial strains aside from E. Coli could have similar effects, we conducted evolutionary experiments on four bacterial strains that included *E. coli* O157:H7, *E. coli* O103:H2, *Pseudomonas putida*, and *Staphylococcus epidermidis*. The four strains were subjected to a coexposure of streptomycin and environmental levels of pesticides that are below the minimal inhibitory concentrations (MIC). The bacterial strains were exposed for 500 generations and changes in their resistance levels were measured and observed. The final bacterial colonies were then sequenced and studied to characterize whether any genetic mutations had occurred and through which mechanism of resistance.

My contribution to this study involved the coexposure of the streptomycin and environmental levels of pesticides that are below the MIC on the *E. coli* O103:H2 bacterial strain. For the purpose of this capstone, I will include data from this study for all four of the bacterial strains tested.

Materials and Methods

Bacterial Strains, Growth, Selection Conditions, and Evolutionary Experiments. The bacterial strains used in this study were purchased from ATCC: The Global Bioresource Center,

including an *Escherichia coli* O157:H7 strain (ATCC No. 43888), an *Escherichia coli* O103:H2 strain (which was kindly received from the Salinity Laboratory of USDA in Riverside), a *Pseudomonas putida* strain (ATCC No. 12633), and a *Staphylococcus epidermidis* strain (ATCC No. 14990). 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 MIC of 7, 8, 4, 8 mg/L, respectively, which were used for the following evolutionary experiments.

The selection conditions included streptomycin-only (Strep-only), pesticide-only, and the coexposure to Strep and pesticides. The selection concentration of Strep is at sub-MIC level (i.e., 1/5 MIC_0) [denoted as (1/5,0)]. The pesticides used in this study were consistent with our previous study[10] (Table S1), which included a variety of pesticides that are frequently detected in the aquatic environments. For the exposure concentrations, we defined environmental concentrations (EC) based on their detection records (0.1 – 4.8 μg/L each and ~20 μg/L in total). We applied three pesticide exposure levels, which are 1, 10, and 100 times of EC, corresponding to the occurrence levels of pesticides at various environmental exposure scenarios [denoted as (0,1), (0,10), (0,100), respectively]. The coexposure conditions thus are combinations of 1/5 MIC_0 and different concentrations of pesticides [denoted as (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 (Figure S1).

Evolutionary experiments were performed as described in our previous study[10]. Briefly, we serially transferred 8 replicate populations for 500 generations in 200 μL LB liquid media containing certain selection conditions in a 96-well plate. The pesticide mixture was prepared in

methanol, added to the wells, and evaporated prior to adding LB media and the Strep stock solution. 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 exposure conditions. Each transfer resulted in log2(500) = ~ 9 generations, and the evolutionary experiments lasted for 56 days (Figure S1). The cultures after every 100 generations were preserved by adding 100 µL of 50% glycerol and stored at −80 ºC.

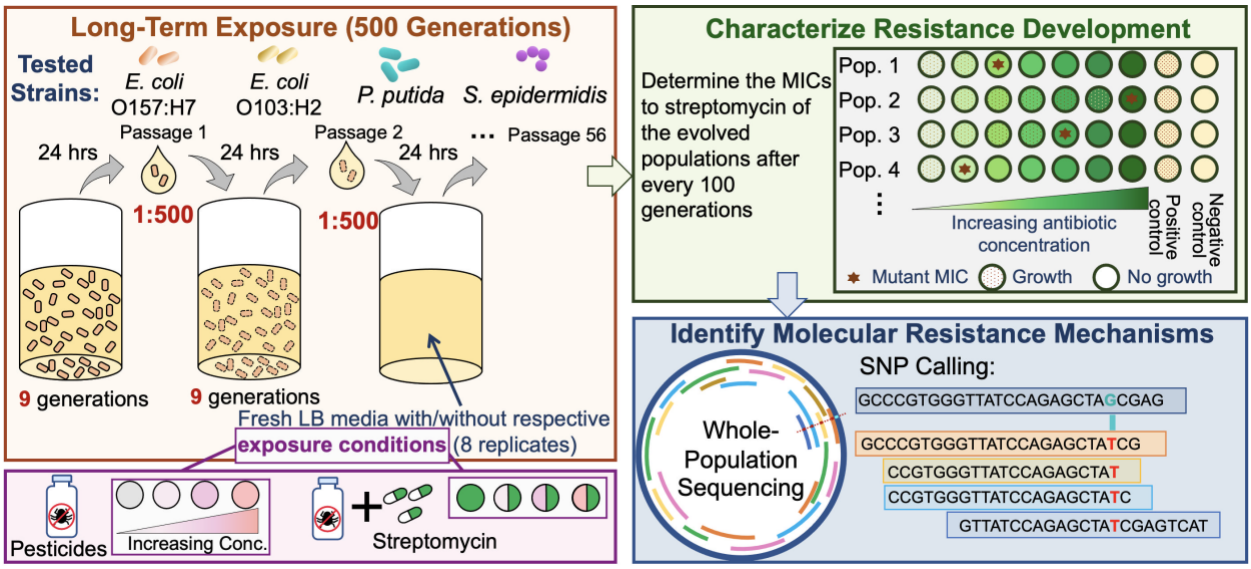

Figure S1. Illustration of experimental design. Three bacterial strains were examined and a total of 8 parallel populations of each strain under each exposure condition were serially passaged every 24 h (dilution factor = 1:500, ~9 generations) into fresh LB medium containing streptomycin and/or pesticides at the same exposure levels for 500 generations. The exposure conditions include pesticides-only: 1EC (environmentally relevant concentrations), 10EC, 100EC; streptomycin-only, the coexposure to both pesticides and streptomycin. The control group without selective pressures was set up in the meanwhile. The resistance levels of evolved populations were

characterized by MIC tests, and several evolved populations were subject to whole-population sequencing to identify resistance mechanisms.

Pesticides	Category	Mode of action	Conc. (μg/L)	Occurring environment
2,4-D	Herbicide	Synthetic plant hormone	0.2	Urban run-off[1]
Mecoprop	Herbicide		2	Urban run-off[1]
Benomyl	Fungicide	Inhibits cell division	0.2	Surface water[2]
Metolachlor	Herbicide		0.4	Wastewater[3]
Thiabendazole	Fungicide		0.2	Wastewater influent[4]
Carbaryl	Insecticide	Acetylchlolin-esterase	4.8	Surface water[5]
Carbofuran	Insecticide	inhibitor; nervous system	0.38	Subsurface and surface water[6]
Chlorpyrifos	Pesticide	disruptor	0.4	Lake[7]
Diazinon	Insecticide		0.3	Wastewater[3]
Fipronil	Insecticide		0.2	Urban surface water[8]
Imidacloprid	Insecticide		0.4	Subsurface and surface water[6]
Propiconazole	Fungicide	Inhibits sterol synthesis	1	Wastewater[9]
Imazalil	Fungicide	and damage membrane	0.4	River[10]
Clotrimazole	Fungicide	permeability	0.1	Wastewater[11]
Irgarol	Biocide	Inhibits photosynthesis	0.2	Coastal water[12]
Linuron	Herbicide		2	Rivers[13]
Diuron	Herbicide		1	Urban run-off[1]
Atrazine	Herbicide		0.5	Subsurface and surface water[14]
Terbuthylazine	Herbicide		0.65	Subsurface and surface water[6]
Terbutryn	Herbicide		0.5	Rivers[15]
Tebuconazole	Fungicide	Inhibits spore spread	0.5	Wastewater[16]
DEET	Biocide	Interferes with neurons and receptors	3	Wastewater influent[17]
Metaldehyde	Pesticide	Produces mucus	0.5	Surface water[18]
		Total	**19.83**	

Table S1. Selected pesticides and their environmental concentrations (EC). The values provided on this table were used to guide the pesticide exposure levels used in our study based on their levels of detection.

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 into fresh LB medium plus 5 μL of nanopore water instead of the antibiotic solution. The negative control was the same as growth control 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 significance of MIC differences of the coexposure conditions and single exposure (p-value < 0.05, N = 8, unpaired, two-tailed, unequal variances).

DNA Extraction and Whole-Population Sequencing. To identify and compare the genetic mutations in the evolved populations of *E. coli* O157:H7 and *E. coli* O103:H2 with Strep resistance development, we sequenced 500-generation populations with Strep-only exposure and the coexposure (two/three replicates for each condition), which have developed increased levels of antibiotic resistance. The evolved populations without chemical exposure were also sequenced to identify genetic adaptations to the growth conditions. The populations under the pesticide-only condition were not sequenced as none of them showed significant resistance development. Each evolved population was cultivated overnight in LB medium, and cell pellets were collected by centrifugation. Genomic DNA (gDNA) was extracted using the DNeasy Blood and Tissue Kit (Qiagen), and the gDNA concentrations were determined on a Qubit 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE). The gDNA was then subjected to 150-bp paired-end sequencing on the Illumina NextSeq platform, which was carried out by Microbial Genome Sequencing Center. The mutant alleles were called out by the workflow described previously.[7,8] A dynamic sequence trimming was done by SolexaQA software[17] with a minimum quality score of 30 and a minimum sequence length of 50 bp. All samples were aligned against the *E. coli* O157:H7 ATCC 43888 genome and *E. coli* O103:H2 genome available at NCBI GenBank (NZ_CP041623.1

and AP010958.1) using the Bowtie 2 toolkit[25]. SAMtools was used to format and reformat the intermediate-alignment files[26]. SNPs and INDELs were identified and annotated with software BCFtools[27] and SnpEff[28]. Among these, the valid mutant alleles were further filtered based on the criteria: (i) causing amino-acid-sequence change, (ii) not found in the ancestor G_0 and the evolved populations without selective pressures at generation 500, (iii) > 20-read coverage, and (iv) > 5% (1/20) mutant allele frequency at the mutation positions.

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. Several *rpsL* mutants were subject to whole-genome sequencing to identify genetic mutations. The SNP calling procedures were the same as the analysis of whole-population sequencing data described above, except for the mutation frequency greater than 50%.

Results and Discussion

Synergistic Effects of Pesticides and Streptomycin on Different Bacterial Populations and the Selection of Antibiotic Resistance. Four different bacterial strain populations were coexposed for 500 generations and each acquired different resistance to the streptomycin application. For the *E. coli* O157:H7 strain (Figure 1A), a strong resistance of a > 40-fold increase in the minimal inhibitory concentration resulted after the coexposure at the three concentrations. High -level resistance was observed in the evolved populations as the pesticide concentrations ranging from 1EC to 100 EC were gradually increased. When comparing these results to the populations who were exposed to the streptomycin alone, these populations exhibited only a mild resistance of 4-10 folds increase compared to its original MIC. *E. coli* O157:H7 populations exposed to the treatment of pesticide alone had little impact on increasing the levels of MIC. When analyzing the results for the *E. coli* O103:H2 strain (Figure 1B) similar developments were observed. Populations of the *E. coli* O103:H2 strain who were coexposed to the pesticide and streptomycin mixture developed strong antibiotic resistance. The resistance observed ranged from 20-50 folds. For the populations exposed to streptomycin alone, any resistance observed was minimal and had a resistance of less than 5 folds. Results from our two bacterial *E. coli* strains studied showed parallel results with other *E. coli* (K-12) strains that were exposed to the same levels of pesticide and antibiotics furthermore highlighting the synergistic effect that the combination has and may have on other strains within the *E. coli* family.[8] Less applicable to my capstone but worth mention is the effects that the coexposure, streptomycin-only, and pesticide-only exposures had on the two other bacterial strains incorporated in the study. For the *P. putida* strain, the only exposure that led to any form of resistance resulted in the strep-only leading to mild resistance of a 4-6-fold in the initial MIC levels in the populations (Figure 1C). For the last strain tested, *S. epidermidis*, the exposure conditions had no impact on

the evolved populations resistance to streptomycin at the conclusion of 500 generations. The levels measured had little to no change compared to their original MIC levels (Figure 1D). From these results we can further correlate that the synergistic effect of pesticides and streptomycin is capable of leading to concerning levels of antibiotic resistance in a variety of *E. coli* strains. Further studies, with different bacterial strains, may need to be conducted in the future to better understand which strains, besides *E. coli* can develop a greater resistance to antibiotics under the same coexposure conditions.

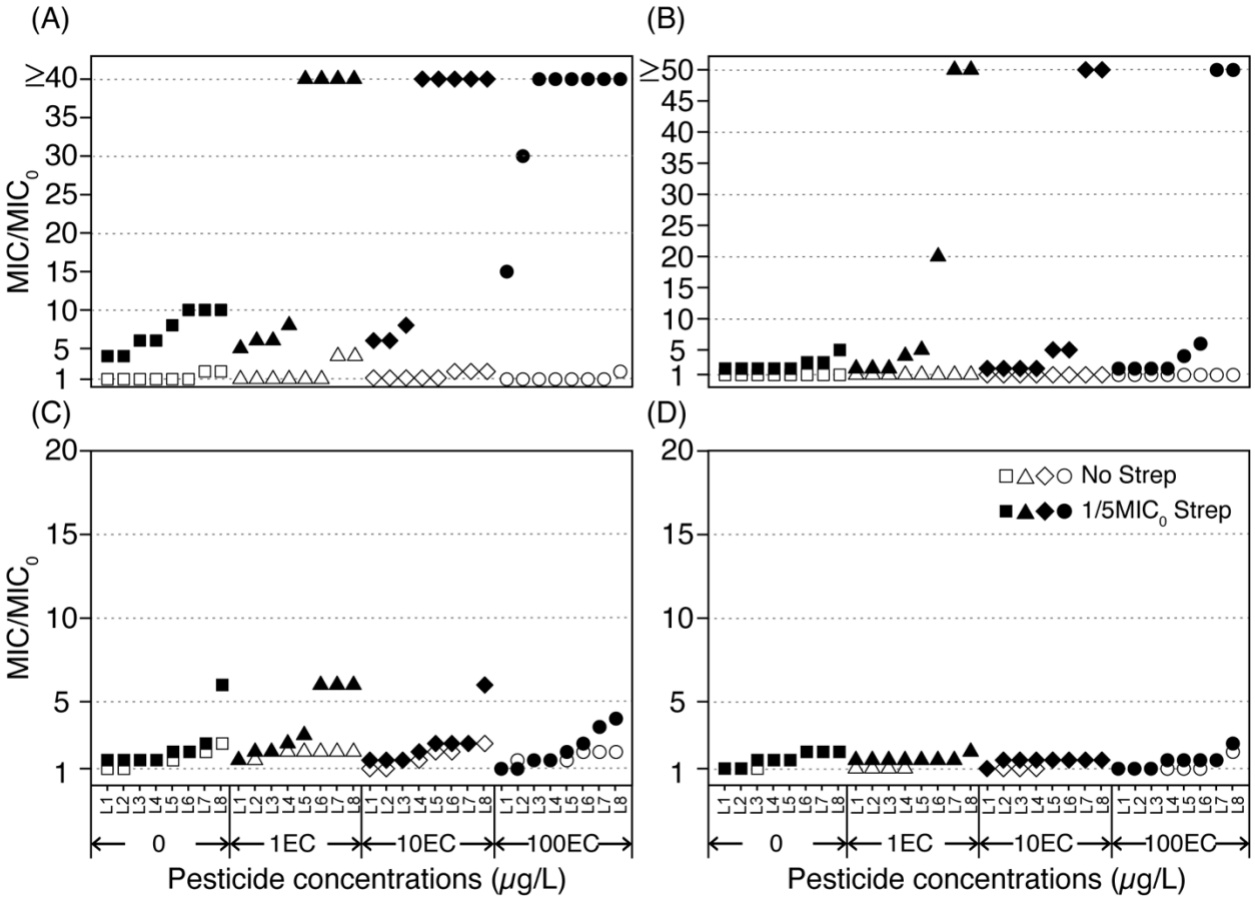

Figure 1. Population MICs of *E. coli* O157:H7 (A), *E. coli* O103:H2 (B), *P. putida* (C), *S. epidermidis* (D) under Strep-only, pesticide-only, and the coexposure conditions after 500-generation evolution (eight parallel lineages, L1-L8, were performed; The MICs of the *E. coli*

O157:H7, *E. coli* O103:H2, *P. putida*, and *S. epidermidis* ancestor strains are 7, 8, 4, and 8 mg/L, respectively).

Mechanisms of Resistance of the Evolved *E. coli* O157:H7 and *E. coli* O103:H2 Populations with Exposure to Different Conditions. To further understand the antibiotic resistance that occurred within our *E. coli* samples, mutations were identified in both the *E. coli* O157:H7 and *E. coli* O103:H2 evolved populations at generation 500. The mutations were identified for the coexposure condition and for the condition containing streptomycin alone with the purpose of comparing genes that had led to the development of a resistance to streptomycin in each of the strains. Our study determined that the evolved *E. coli* O157:H7 populations under the coexposure condition followed resistance mechanisms dependent on target modification, stress response, substrate uptake, fimbriae, flagella, and motility, phage, and metabolism (figure 3A). Mutated genes observed in the evolved populations under the streptomycin only condition, experienced resistance mechanisms in stress response, substrate uptake, phage, and metabolism (Figure 3A). In the coexposure for *E. coli* O103:H2 populations, mutations were observed in genes found in target modification, DNA replication and transcription, stress response, substrate uptake, fimbriae, flagella, and motility, and metabolism (Figure 3B). For the exposure of the *E. coli* O103:H2 populations to streptomycin alone, gene mutations were detected in target modification, stress response, substrate uptake, fimbriae, flagella, and motility, and lastly phage (Figure 3B).

When looking at the exact mutations that took place within the two *E. coli* strains studied, there were some similarities. Under both the coexposure conditions and the exposure to streptomycin alone, mutations mutually occurred in the genes *cpxA* and *sbmA*. This further helps

to link the similar behaviors between the two *E. coli* strains since the mutations occurred through the same mechanisms of resistance. Crucial to understanding the resistance mechanisms that occurred is taking a better look at the CpxA and CpxR systems. When a mutation occurs within the *cpxA* gene, that directly triggers the Cpx stress response which in turn allows for resistance to antibiotics within bacterial species.[10] Both previously mentioned systems, are responsible for the sensing and responding to periplasmic stress, which in turn will contribute greatly to the level of antibiotic resistance observed in different bacterial strains. [10,11] Mutations in the gene *cpxA* have been addressed in previous studies. Through evolutionary experiments conducted these studies once again concluded that a mutation within the *cpxA* gene had led to the resistance of bacteria to beta-lactams and aminoglycosides.[12] The mutations that occurred within our *cpxA* gene in both our *E. coli* samples, did not agree with those observed in other studies but we believe it may have followed a similar resistance mechanism. Further mutations were identified in the *sbmA* gene that were linked to missense mutations, frameshift mutations, and stop- gained mutations (Table S2, Figure 3). These mutations can be correlated to the loss of function exhibited in SbmA who functions as a antimicrobial peptide transporter. In a pervious study conducted, findings showed that a mutation in that particular gene, was linked to an enhanced resistance of *E. coli* to streptomycin.[11] This previous finding can be applied to our current study as the possible resistance mechanism that occurred in *sbmA* (Figure 4).

The phenotypic resistance observed in our bacteria strains gained its resistance through the previously mentioned genetic mutations. These mutations were observed in the coexposure conditions but failed to be observed under the streptomycin only conditions. In the bacterial strain *E. coli* O157:H7, the specific mutations observed were in the *rpsL* genes. The specific mutations that occurred within rpsL were Lys88Gln, Lys88Arg, and Lys88Thr (Figure 3A). The

purpose of the *rpsL* gene is to encode for the S12 ribosomal protein which is the primary target of streptomycin.[24] In multiple bacterial strains, streptomycin resistance was commonly observed in position 88 of Lys88Arg and Lys88Gln due to mutations from single-amino-acid-substitution.[13-16] Aside from the mutations observed in the *rpsL* genes, under the coexposure conditions, further mutations occurred in the *dliM* and *mot* genes relating to cell motility and in the gene *FNZ21_RS20200* involved with phage proteins (Figure 3A).

The results for the *E. coli* O103:H2 bacterial strain again exhibited mutations in *rspL*. The genes particularly impacted were Lys43Arg, Lys43Asn, and Arg86Ser in the coexposed populations. The populations that exhibited these mutations, showed high resistance. Similarly, in a study conducted for an *E. coli* K-12 population where the bacteria were exposed to the same pesticides as those utilized in this study, the *E. coli* K-12 population also contained a mutation in the *rpsL* gene (Arg86Ser) (Figure 4).[11] Mutations for this strain also occurred in *mutT* and *mutL*, mutator genes (Figure 3B). These mutations may be responsible for the increase in mutation frequency and other mutator phenotypes that occur. Mutators have been studied and are known to cause either high or minimal antibiotic resistance because of their increase in selectivity of mutants.[17] When these types of mutations occur, it reassures the idea that a coexposure of pesticides and streptomycin on bacterial strains, in this case *E.* coli, have the capability of increasing the number and frequency at which mutations occur.

Table S2. List of valid mutant alleles identified in the evolved populations of *E. coli* O157:H7 and *E. coli* O103:H2

Evolved Populations	Gene	Genome position	Nucleotide change	Type of mutation	Amino acid change	Mutant allele frequency
***E. coli* O157:H7**						

(1/5,0)-L6	*cpxA*	902049	T → A	Missense variant	Val94Glu	1
	yejF	2794038	G → T	Stop gained	Ser44*	0.43
	yejB	2795474	AT → A	Frameshift variant	Asn272fs	0.3
	yejB	2795872	C → T	Missense variant	Gly140Arg	0.19
	sbmA	5237151	C → T	Stop gained	Trp99*	1
	dmsA_ynfE	2441508	C → G	Missense variant	Pro204Ala	0.18
	FNZ21_RS17055	3455726	T → C	Missense variant	Leu4Ser	0.26
	FNZ21_RS17055	3455729	T → G	Missense variant	Ile5Ser	0.25
(1/5,0)-L7	*cpxA*	902522	A → C	Missense variant	Thr252Pro	0.96
	dmsA_ynfE	2441508	C → G	Missense variant	Pro204Ala	0.36
	dmsA_ynfE	2441509	C → G	Missense variant	Pro204Arg	0.14
	yejF	2793772	C → A	Stop gained	Glu133*	0.27
	yejA	2796600	G → T	Stop gained	Ser502*	0.47
	FNZ21_RS17055	3455726	T → C	Missense variant	Leu4Ser	0.31
	FNZ21_RS17055	3455729	T → G	Missense variant	Ile5Ser	0.29
	sbmA	5237151	C → T	Stop gained	Trp99*	0.96
(1/5,0)-L8	*FNZ21_RS27775*	13160	G → C	Missense variant	Leu8Val	0.19
	FNZ21_RS27775	13164	T → TA	Frameshift variant	Glu6fs	0.15
	FNZ21_RS27775	13170	A → AT	Frameshift variant	Ala4fs	0.07
	cpxA	901880	C → T	Missense variant	Leu38Phe	0.56
	cpxA	902034	C → T	Missense variant	Thr89Ile	0.42
	rpsL	1005586	A → T	Missense variant	Leu49Gln	0.23
	dmsA_ynfE	2441508	C → G	Missense variant	Pro204Ala	0.32
	dmsA_ynfE	2441509	C → G	Missense variant	Pro204Arg	0.07
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.33
	yejE	2794742	GAGTCAGC ATCAGG → G	Frameshift variant	Gly147fs	0.56
	yejE	2794742	G → GC	Frameshift variant	Leu152fs	0.13

	yejE	2794756	C → G	Missense variant	Gly147Ala	0.1
	FNZ21_RS17055	3455726	T → C	Missense variant	Leu4Ser	0.29
	FNZ21_RS17055	3455729	T → G	Missense variant	Ile5Ser	0.26
	ydeQ	3717517	T → G	Missense variant	Leu81Arg	0.28
	sbmA	5236674	A → T	Missense variant	Leu258Gln	0.78
(1/5,1)-L6	*cpxA*	902034	C → T	Missense variant	Thr89Ile	0.95
	rpsL	1005469	T → C	Missense variant	Lys88Arg	0.05
	rpsL	1005586	A → T	Missense variant	Leu49Gln	0.72
	yejE	2794648	C → T	Stop gained	Trp183*	0.2
	ymdA	3279177	T → A	Missense variant	Met10Lys	0.29
	ymdA	3279178	C → G	Missense variant	Met10Ile	0.3
	ymdA	3279180	T → G	Missense variant	Leu11Arg	0.22
	ymdA	3279182	G → C	Missense variant	Gly12Ala	0.22
	ymdA	3279185	A → G	Missense variant	Ser13Gly	0.22
	ydeQ	3717517	T → G	Missense variant	Leu81Arg	0.73
	sbmA	5236959	A → T	Missense variant	Val163Glu	0.36
(1/5,1)-L7	*cpxA*	902034	C → T	Missense variant	Thr89Ile	0.18
	rpsL	1005469	T → C	Missense variant	Lys88Arg	0.76
	rpsL	1005586	A → T	Missense variant	Leu49Gln	0.18
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.3
	ydeQ	3717517	T → G	Missense variant	Leu81Arg	0.21
	fliM	4460129	C → T	Missense variant	Arg181Cys	0.69
	sbmA	5236894	C → A	Stop gained	Glu185*	0.21
(1/5,1)-L8	*cpxA*	902034	C → T	Missense variant	Thr89Ile	0.62
	rpoA	986366	G → A	Missense variant	Arg317Cys	0.26
	rpsL	1005586	A → T	Missense variant	Leu49Gln	0.53
	ydeQ	3717517	T → G	Missense variant	Leu81Arg	0.46

(1/5,10)-L6	*rpsL*	1005469	T → C	Missense variant	Lys88Arg	0.99
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.28
	FNZ21_RS20200	4036009	G → A	Missense variant	Ala720Val	1
(1/5,10)-L7	*FNZ21_RS27775*	13160	G → C	Missense variant	Leu8Val	0.2
	rpsL	1005469	T → C	Missense variant	Lys88Arg	1
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.32
	motB	4392507	C → G	Missense variant	Trp211Cys	0.19
	fliM	4460129	C → T	Missense variant	Arg181Cys	0.52
(1/5,10)-L8	*FNZ21_RS27775*	13160	G → C	Missense variant	Leu8Val	0.22
	rpsL	1005469	T → C	Missense variant	Lys88Arg	0.99
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.37
	FNZ21_RS20200	4036009	G → A	Missense variant	Ala720Val	0.99
	cheZ	4383813	C → CA	Frameshift variant	Asp38fs	0.21
	motA	4393344	A → C	Stop gained	Leu227*	0.36
(1/5,100)-L6	*FNZ21_RS00045*	5208	A → AT	Frameshift variant	Asn180fs	0.92
	FNZ21_RS00045	5394	C → CA	Frameshift variant	Leu118fs	0.98
	FNZ21_RS27775	13160	G → C	Missense variant	Leu8Val	0.15
	FNZ21_RS27775	13164	T → TA	Frameshift variant	Glu6fs	0.11
	FNZ21_RS27775	13170	A → AT	Frameshift variant	Ala4fs	0.06
	cpxA	902676	T → G	Missense variant	Leu303Arg	0.2
	rpsL	1005469	T → C	Missense variant	Lys88Arg	0.82
	rpsL	1005469	T → G	Missense variant	Lys88Thr	0.18
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.25
	FNZ21_RS20200	4036009	G → A	Missense variant	Ala720Val	0.22
	FNZ21_RS00045	5208	A → AT	Frameshift variant	Asn180fs	0.95

(1/5,100)-L7	*FNZ21_RS00045*	5394	C → CA	Frameshift variant	Leu118fs	0.99
	FNZ21_RS27775	13160	G → C	Missense variant	Leu8Val	0.19
	FNZ21_RS27775	13164	T → TA	Frameshift variant	Glu6fs	0.15
	FNZ21_RS27775	13170	A → AT	Frameshift variant	Ala4fs	0.07
	cpxA	902018	C → T	Missense variant	Arg84Cys	0.94
	rpsL	1005469	T → C	Missense variant	Lys88Arg	0.07
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.15
	yejF	2794038	G → T	Stop gained	Ser44*	0.32
	yejA	2797068	C → T	Stop gained	Trp346*	0.32
	yejA	2797213	C → A	Stop gained	Glu298*	0.14
	sbmA	5236311	A → ATG	Frameshift variant	Leu379fs	0.37
	sbmA	5236311	AAC → A	Frameshift variant	Leu379fs	0.16
(1/5,100)-L8	*FNZ21_RS00045*	5208	A → AT	Frameshift variant	Asn180fs	0.92
	FNZ21_RS00045	5394	C → CA	Frameshift variant	Leu118fs	0.98
	FNZ21_RS27775	13160	G → C	Missense variant	Leu8Val	0.19
	FNZ21_RS27775	13164	T → TA	Frameshift variant	Glu6fs	0.15
	FNZ21_RS27775	13170	A → AT	Frameshift variant	Ala4fs	0.07
	cpxA	902018	C → T	Missense variant	Arg84Cys	0.25
	cpxA	902676	T → G	Missense variant	Leu303Arg	0.67
	rpsL	1005469	T → G	Missense variant	Lys88Thr	0.62
	rpsL	1005470	T → G	Missense variant	Lys88Gln	0.17
	dmsA_ynfE	2441529	T → G	Missense variant	Val211Gly	0.21
***E. coli* O103:H2**						
(1/5,0)-L6	*gidB*	4540629	C → T	Missense variant	Pro79Leu	0.35
	cpxA	4744717	T → A	Missense variant	Val94Glu	0.74
	ECO103_5189	5382329	A → ATC	Frameshift variant	Ile5fs	0.1
	sbmA	403385	C → A	Missense variant	Asn109Lys	1

(1/5,0)-L7	*ymdA*	1190258	G → T	Missense variant	Gly35Cys	0.79
	flgG	1219513	T → G	Missense variant	Leu26Arg	0.2
	yejA	2685839	G → A	Stop gained	Trp566*	0.84
	cpxA	4744687	G → C	Missense variant	Arg84Pro	0.13
	ECO103_0218	250358	A → AC	Frameshift variant	Ile140fs	0.06
	yaiT	398230	G → GAACCGCC	Frameshift variant	Ser124fs	0.05
	ECO103_1873	1964419	G → GCGTTAT	Conservative inframe insertion	Glu11_Ala12ins ArgTyr	0.05
(1/5,0)-L8	*dnaJ*	15624	C → A	Stop gained	Ser60*	0.48
	ECO103_2106	2181602	C → CGAGCAT	Conservative inframe insertion	Ser47_Ala48ins MetLeu	0.15
	fliC	2244294	C → G	Missense variant	Ala139Pro	0.27
	rpsL	4135948	G → A	Missense variant	Pro91Ser	0.88
	gidB	4540832	ATGGTGAGC TGGTGTCAC CATCTTCC → A	Frameshift variant	Trp150fs	0.75
	yjjK	5437633	T → A	Missense variant	Glu202Val	0.32
	yaiT	398228	A → ATG AACCGCCCC	Frameshift variant & stop gained	Ser124fs	0.06
	gidB	4540840	C → CAAGG	Frameshift variant	Trp150fs	0.05
	ECO103_5181	5377894	TTC → T	Frameshift variant	Leu73fs	0.09
(1/5,1)-L6	*iscR*	3104852	T → TCTCA	Frameshift variant	Asp102fs	0.12
	hybF	3753160	T → C	Missense variant	Ile33Val	0.94
	rpsL	4135963	G → T	Missense variant	Arg86Ser	1
	gidB	4540632	G → A	Missense variant	Gly80Asp	0.92
	proQ	2104319	CGCGGT → C	Frameshift variant	Arg137fs	0.23

(1/5,1)-L7	*hybF*	3753160	T → C	Missense variant	Ile33Val	1
	rpsL	4135963	G → T	Missense variant	Arg86Ser	0.99
	gidB	4540632	G → A	Missense variant	Gly80Asp	1
	ECO103_3642	3715176	A → ATCA	Disruptive inframe insertion	Arg19_Thr20ins Ile	0.07
(1/5,1)-L8	*sbmA*	403148	T → TG	Frameshift variant	Glu33fs	0.22
	sbmA	403590	T → A	Missense variant	Trp178Arg	0.71
	ycdU	1181379	A → G	Missense variant	Gln84Arg	0.15
	flhD	2220208	A → G	Missense variant	Ile10Thr	0.14
	ECO103_3664	3740777	G → A	Missense variant	Thr172Ile	0.18
	yheS	4143171	A → G	Missense variant	Thr73Ala	0.16
	gidB	4540518	C → A	Stop gained	Ser42*	0.76
	cpxA	4745344	T → G	Missense variant	Leu303Arg	1
	mutL	5155663	A → AGCTGGC	Disruptive inframe insertion	Leu72_Ala73dup	0.12
	fimE	5296962	C → T	Stop gained	Gln17*	1
(1/5,10)-L7	*rpsL*	4136091	T → C	Missense variant	Lys43Arg	0.97
	leuX	5256669	CG → C	Frameshift variant	Arg2fs	0.23
	leuX	5256670	G → GCACCT TCGCACCTTC	Conservative inframe insertion	Cys1_Arg2insGlu GlyAlaLysVal	0.41
(1/5,10)-L8	*flhD*	2219948	A → G	Missense variant	Ser97Pro	0.23
	amyA	2248635	C → T	Missense variant	Arg465Trp	0.19
	rpsL	4136090	T → G	Missense variant	Lys43Asn	0.22
	rpsL	4136091	T → C	Missense variant	Lys43Arg	0.77
	leuX	5256670	G → GCACCT TCGCACCTTC	Conservative inframe insertion	Cys1_Arg2insGlu GlyAlaLysVal	0.39
	ECO103_5071	5274480	C → A	Missense variant	Pro540Thr	0.17

(1/5,100)-L7	*pgsA*	2233949	A → T	Missense variant	Val44Glu	0.99
	ECO103_4555	4678192	C → A	Missense variant	Thr268Asn	0.26
	ECO103_5192	5383961	C → T	Missense variant	Thr158Ile	0.28
(1/5,100)-L8	*mutT*	112921	T → G	Missense variant	Leu54Arg	0.38
	acnB	135636	T → G	Missense variant	Asp839Glu	0.3
	mrcB	167185	T → G	Missense variant	Met353Arg	0.32
	fadE	275259	T → G	Missense variant	Ser22Arg	0.48
	trxB	1016359	T → G	Missense variant	Asn98His	0.34
	trxB	1016565	T → G	Missense variant	Asn29Thr	0.12
	yncD	1666674	A → C	Missense variant	Asn219Lys	0.3
	yebT	2108979	T → G	Stop gained	Leu760*	0.28
	yebA	2129931	A → C	Missense variant	Phe299Cys	0.49
	napA	2712072	T → G	Missense variant	Asn363His	0.12
	yfgH	3072962	A → C	Missense variant	Ile49Leu	0.2
	yhbH	4030505	C → CATAAAG	Conservative inframe insertion	Asp90_Lys91dup	0.27
	rpsL	4136091	T → C	Missense variant	Lys43Arg	0.17
	cpxA	4744987	G → T	Missense variant	Trp184Leu	0.36
	xylG	4805796	A → C	Missense variant	Ser209Ala	0.51

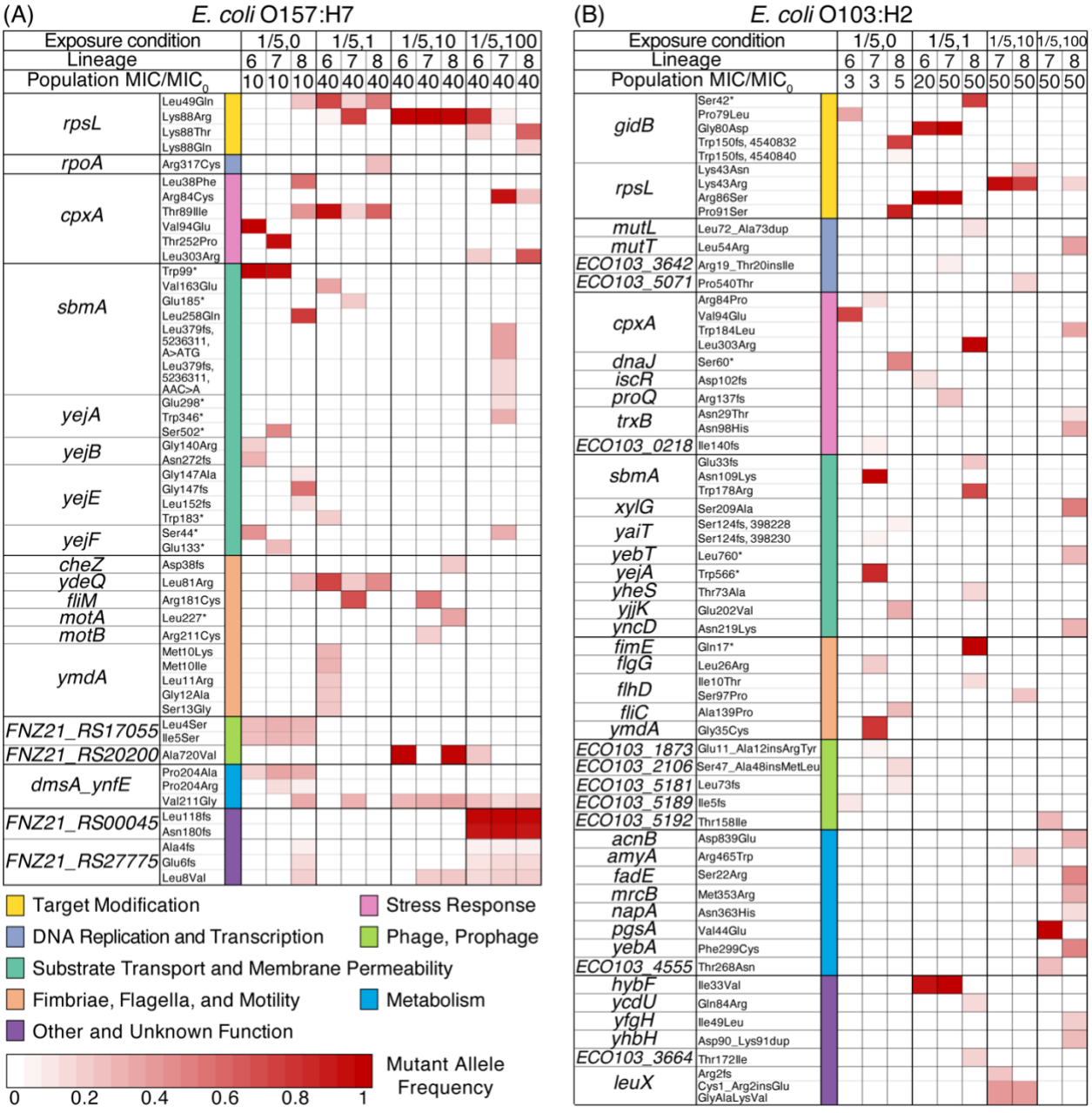

Figure 3. Heatmap of the mutant allele frequency of genetic mutations identified in the evolved populations with the coexposure and the Strep-only exposure in *E. coli* O157:H7 (A) and *E. coli* O103:H2 (B).

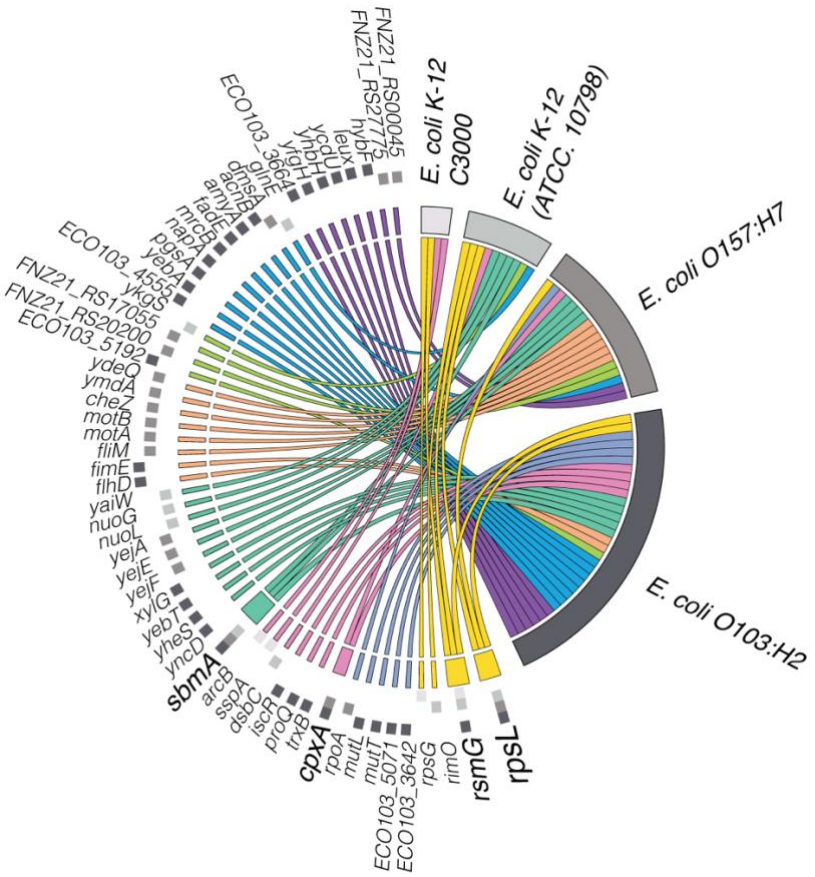

Figure 4. Summary of identified mutations conferring streptomycin resistance in different *E. coli* strains exposed to pesticides.

Conclusion. The results from this study are an important contribution to the ongoing studies conducted under the topic of antibiotic resistance evolution in the environment. One of the most important findings from this study is recognizing that the combination of pesticides and streptomycin will not always have the same synergistic effect on all bacterial genera.

In this study conducted with multiple bacterial strains this synergistic effect of pesticides and streptomycin was observed in the *E. coli* strains (the O157:H7 strain and the O103:H2 strain). This study we conducted is part of ongoing studies conducted by a graduate student, Yue Xing, part of the Department of Chemical and Environmental Engineering at the University of California, Riverside. In her previous studies, she had already determined that the *E. coli* K-12

C3000 and *E. coli* K-12 ATCC. 10798 had also experienced a similar synergistic effect with the combination of pesticides and streptomycin (Figure 4).[7,8] It is highly likely that this synergistic effect only impacts certain bacteria like *E. coli.* The use of the strains *E. coli* O157:H7 and O103:H2 strain provide important results and clinical relevance because they have already been acknowledged as the cause for many diarrheal illness outbreaks.[18-23] Resistant phenotypes in these strains are of concern and should be monitored closely. Following the coexposure of pesticides and streptomycin on the two *E. coli* strains, their increased resistance is even more of a threat to public health. If these pathogenic *E. coli* strains happen to be present in an environment while at the same time as antibiotics and pesticides, the resistance observed during our study could also be a possible environmental observation. Antibiotic resistance in these strains has a greater possibility of being observed in agriculture, wastewater treatment plants, and surface water bodies that receive drainage from agricultural runoff because application of pesticides and antibiotics are highly used in the agriculture industry. Extensive studies are still needed to better understand how environmentally isolated *E. coli* strains will gain resistance to certain antibiotics. Another important finding from this study was the identification of the mutations that occurred in the *rpsL* genes associated with target modification in the *E. coli* microbial populations that were exposed to both the pesticides and antibiotics. The mutations in the *rspL* genes led to increased levels of phenotypic resistance. Through this study, resistance mechanisms like, *cpxA* and *sbmA,* were also identified under the conditions of coexposure and streptomycin only. It is possible that the resistance mechanisms detected may be convergent even if the bacteria strain who has gained resistance is put under different selective pressures. If convergent, this would allow for the potential detection of specific genetic markers to analyze for antibiotic resistance levels. Just as the *rpsL* mutation, Lys88Arg, leads to high levels of phenotypic resistance, it is possible that

specific single-amino-acid-alteration could also experience similar effects and have high phenotypic resistance. Detecting mutations in bacterial strains whether it be gene or phenotypic resistance, will be crucial for preventing the spread of pathogenic bacteria that may be of harm to the environment and humans.

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