Alterations of *Salmonella enterica* Serovar Typhimurium Antibiotic Resistance under Environmental Pressure

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ABSTRACT Microbial horizontal gene transfer is a continuous process that shapes bacterial genomic adaptation to the environment and the composition of concurrent microbial ecology. This includes the potential impact of synthetic antibiotic utilization in farm animal production on overall antibiotic resistance issues; however, the mechanisms behind the evolution of microbial communities are not fully understood. We explored potential mechanisms by experimentally examining the relatedness of phylogenetic inference between multidrug-resistant *Salmonella enterica* serovar Typhimurium isolates and pathogenic *Salmonella* Typhimurium strains based on genome-wide single-nucleotide polymorphism (SNP) comparisons. Antibiotic-resistant *S. Typhimurium* isolates in a simulated farm environment barely lost their resistance, whereas sensitive *S. Typhimurium* isolates in soils gradually acquired higher tetracycline resistance under antibiotic pressure and manipulated differential expression of antibiotic-resistant genes. The expeditious development of antibiotic resistance and the ensuing genetic alterations in antimicrobial resistance genes in *S. Typhimurium* warrant effective actions to control the dissemination of *Salmonella* antibiotic resistance.

IMPORTANCE Antibiotic resistance is attributed to the misuse or overuse of antibiotics in agriculture, and antibiotic resistance genes can also be transferred to bacteria under environmental stress. In this study, we report a unidirectional alteration in antibiotic resistance from susceptibility to increased resistance. Highly sensitive *Salmonella enterica* serovar Typhimurium isolates from organic farm systems quickly acquired tetracycline resistance under antibiotic pressure in simulated farm soil environments within 2 weeks, with expression of antibiotic resistance-related genes that was significantly upregulated. Conversely, originally resistant *S. Typhimurium* isolates from conventional farm systems lost little of their resistance when transferred to environments without antibiotic pressure. Additionally, multidrug-resistant *S. Typhimurium* isolates genetically shared relevancy with pathogenic *S. Typhimurium* isolates, whereas susceptible isolates clustered with nonpathogenic strains. These results provide detailed discussion and explanation about the genetic alterations and simultaneous acquisition of antibiotic resistance in *S. Typhimurium* in agricultural environments.

KEYWORDS *Salmonella*, acquired resistance, virulence, soil environment, horizontal gene transfer

More than 20,000 potential antibiotic resistance genes of nearly 400 different types are predicted or identified in available bacterial genome sequences, and this number is expected to increase with further advancements in microbiome analysis (1, 2). Several recent independent studies found that many antibiotic resistance genes that are integrated into the bacterial genomes carry their origins in disparate reservoirs (3,

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4). Furthermore, the acquisition or alteration of those multidrug resistance genes indicates the widespread dissemination of distinct antibiotic resistance genes in various microbial ecosystems, including the intestinal tracts of birds and other species of wildlife (5, 6). It is now well known that horizontal gene transfer is a continuous process and that it shapes bacterial genomes. The influences of microbial ecological niches and the human introduction of synthetic antimicrobials to the microbial ecosystem on the evolution of biological networks are not completely understood.

Animal agriculture, particularly poultry and swine farming in developed countries, accounts for more than half of the antibiotics used in the world (7, 8). Antibiotics are used in animal farming both for treating diseases (therapeutic) and for growth promotion (subtherapeutic) (9). Subtherapeutic dosages of antibiotics in animal feed/water induce faster weight gain via inhibition/modulation of the normal microbiota, leading to increased nutrient utilization and a reduction in the maintenance costs of the gastrointestinal (GI) system due to decreased inflammation (10, 11). Furthermore, subtherapeutic dosage of antibiotics makes additional contributions to animal agriculture by improving animal health and welfare and control of human foodborne infectious diseases by reducing cross-contamination and extending the shelf life of products (9, 12).

In addition to subtherapeutic dosages of antibiotics, significant amounts of therapeutic antibiotics are used in food animals to treat clinical diseases or, prophylactically, to prevent common diseases (9). So far, very little attention, other than the data generated from *in vitro* laboratory studies, has been paid to how dosages of antibiotics used in farm animal production contribute to the overall problem of antibiotic resistance (8, 13). Furthermore, farmers also use various drugs for prevention and treatment of animal parasitic disease; for instance, amprolium (Corid) and sulfadimethoxine are commonly used to control coccidiosis (caused by protozoa of the genus *Eimeria*) in poultry production (14). However, the mechanisms by which the residues of these therapeutic and subtherapeutic antibiotics and other antimicrobials affect horizontal gene transfer have not been evaluated yet.

As current animal farming practices lead to an increase in antibiotic-resistant fecal bacteria in animals, including on carcasses (15–18), and as there is growing concern over antibiotic resistance in human-pathogenic bacteria, the U.S. Food and Drug Administration (FDA) has announced its plan to gradually withdraw nontherapeutic/growth-promoting antibiotics from farm animal production (19–21). Furthermore, consumer desire to avoid synthetic antibiotics has led to increased marketing of meat and poultry from animals reared on organic farms. However, the lower growth rate of organically reared farm animals and their higher mortality rates cause high production costs and nonprofitable practices, as well as higher rates of contamination with zoonotic pathogens that cause foodborne infections (22–24). It is well known that pasture/organic farming cannot meet the demand for animal products for seven billion people. Furthermore, solely blocking or withdrawing subtherapeutic antibiotic use in animal farming without controlling therapeutic or higher doses of antibiotics and use of other chemicals might not be enough to mitigate the threat of rising rates of antibiotic-resistant zoonotic pathogenic bacteria (9, 25, 26). According to published data from the Centers for Disease Control and Prevention (CDC), multidrug resistance in *Salmonella* accounts for approximately 6,200 cases (10% of total) of *Salmonella* infections each year in the United States. *Salmonella enterica* serovar Typhimurium is one of the primary serotypes responsible for antibiotic resistance and among the predominant isolates responsible for multidrug resistance in *Salmonella* infections (27). In the current study, we investigate induced alterations in tetracycline (TET) and sulfamethoxazole-trimethoprim (SXT) resistance and antibiotic resistance-related gene expression for *Salmonella enterica* serovar Typhimurium, both *in vitro* and in soil environments under pressure imposed by these antibiotics, and further characterize derived *S. Typhimurium* isolates based on genome-wide single-nucleotide polymorphism (SNP) comparisons with published pathogenic/nonpathogenic *S. Typhimurium* strains.

TABLE 1 *S. Typhimurium* isolates across distinct categories isolated from farms and markets

Harvest level	Farm/market type	Total no. of isolates	No. of strains analyzed ^e
Preharvest	Mixed crop-livestock farm ^a	25	15
	Conventional poultry farm ^b	3	1
Postharvest	Farmers' market ^c	73	34
	Conventional retailer ^d	7	4
Total		108	54

^aSamples from mixed crop-livestock farms include compost, feces, feed, produce, and soil.^bSamples from conventional poultry farms include chicken bedding.^cSamples from farmers' markets include produce, whole chicken, and chicken parts.^dSamples from conventional retailers include whole chicken and chicken parts.^eThe number of analyzed strains was determined based on the total number of isolates harvested.

RESULTS

***In vitro* antibiotic resistance alterations in *S. Typhimurium*.** To investigate the loss of spontaneous antibiotic resistance, bacterial cells were allowed to grow in optimal culture conditions without antibiotic stress (see Tables 1 and 2 for summaries of isolate sources, distribution, and antibiotic resistance profiles). The initial TET and SXT MICs for both ST-CRC26 and ST-CRC32 isolates were 64 $\mu\text{g/ml}$ and 76/4 $\mu\text{g/ml}$, respectively (Table 3). Twelve passages of both isolates were performed in nutritious blood broth in the absence of antibiotics or any other antimicrobial component. The MIC of TET for the ST-CRC26 isolate was reduced to 32 $\mu\text{g/ml}$, a level that was still above the breakpoint of sensitivity. The MIC of TET for the ST-CRC32 isolate was unchanged (Table 3). Furthermore, MICs of SXT for both isolates ST-CRC26 and ST-CRC32 remained unchanged after 12 passages in this *in vitro* study (Table 3).

Conversely, the non-antibiotic-resistant strains were cultured in antibiotic-stress medium with a double-concentrated dose of antibiotic per two passages to assess the acquisition of antibiotic resistance. The initial TET and SXT MICs for both ST-FMC22 and ST-FMC46 isolates were 4 $\mu\text{g/ml}$ and 19/1 $\mu\text{g/ml}$, respectively (Table 4). During subsequent subculturing, the MICs of these two *S. Typhimurium* isolates were gradually increased until they reached the resistance breaking points of TET and SXT. For TET, ST-FMC22 and ST-FMC46 reached the resistance breakpoint (32 $\mu\text{g/ml}$) after 10 passages, and the TET MIC for ST-FMC46 increased to 64 $\mu\text{g/ml}$ after 12 passages but did not tolerate higher concentrations (Table 4). Similarly, both ST-FMC22 and ST-FMC46 isolates acquired SXT resistance at the 11th passage, but the MIC did not exceed the resistance breakpoint (76/4 $\mu\text{g/ml}$) after 12 passages (Table 4).

Phylogenetic inference and genomic characteristics of *S. Typhimurium* isolates. The relatedness among the *S. Typhimurium* isolates from this study was investigated based on genome-wide SNP comparisons, and the draft genome contigs have been submitted to the NCBI Sequence Read Archive (SRA; accession numbers presented in Table 5). SNPs were identified with the CSI Phylogeny webserver, separating the 54 *S. Typhimurium* isolates from one another and from selected *S. Typhimurium*

TABLE 2 Antimicrobial resistance profiles of *S. Typhimurium* isolates from various sources

Antibiotic susceptibility ^a	Farm/market type (% [no./total])		
	Conventional	Organic	Total
Ampicillin	0.00 (0/5)	2.04 (1/49)	1.85 (1/54)
Cefazolin	0.00 (0/5)	6.12 (3/49)	5.56 (3/54)
Sulfamethoxazole-trimethoprim	80.00 (4/5)	46.94 (23/49)	50.00 (27/54)
Tetracycline	60.00 (3/5)	10.20 (5/49)	14.81 (8/54)
AMR	80.00 (4/5)	65.31 (32/49)	66.67 (36/54)
MDR	60.00 (3/5)	0.00 (0/49)	5.56 (3/54)

^aAMR, antimicrobial resistant (isolates were resistant to at least one antimicrobial tested); MDR, multidrug resistant (isolates were resistant to two or more antimicrobials tested).

TABLE 3 Alterations of antibiotic resistance patterns in *S. Typhimurium* isolates

Strain	MIC ($\mu\text{g/ml}$) for:	
	Sulfamethoxazole-trimethoprim ^a	Tetracycline ^b
ST-CRC26	76/4	64
ST-CRC32	76/4	64
ST-FMC22	19/1	4
ST-FMC46	19/1	4
ST-CRC26m ^c	76/4	32
ST-CRC32m	76/4	64
ST-FMC22m	76/4	32
ST-FMC46m	76/4	64

^aThe resistance breakpoint of sulfamethoxazole-trimethoprim is 76/4 $\mu\text{g/ml}$.

^bThe resistance breakpoint of tetracycline is 32 $\mu\text{g/ml}$.

^cm, mutant strain naturally developed *in vitro*.

published genome sequences available in NCBI GenBank (Fig. 1 and Table 5). According to the whole-genome SNP comparisons, 4 major lineages were identified, in which *S. Typhimurium* isolates from conventional chicken (ST-CRC26, ST-CRC29, ST-CRC31, and ST-CRC32) sources clustered with published pathogenic *S. Typhimurium* strains within lineage 1. Lineage 2 had 3 isolates; ST-FMC71 and ST-O65 (sourced from chicken from organic retailer and farmers' market) clustered with published *S. Typhimurium* isolate ST-U288. In lineages 3 and 4, isolates from organic retailers and farmers' markets clustered with ST-LT2. Assembled whole-genome analysis indicated that the draft genomes of the *S. Typhimurium* isolates from this study harbored plasmids, and the respective NCBI accession numbers of the draft genome sequences are presented in Table 5. Isolates from organic retailers and farmers' markets harbored plasmids IncFIB(S) and IncFII(S). Interestingly, ST-LT2 also harbored plasmids IncFIB(S) and IncFII(S). Isolates from conventional retail chickens, as well as a previously isolated pathogenic ST-21B isolate, harbored plasmids IncA/C2 and ColRNAI. Annotation of the assembled contigs of ST-FMC22, ST-FMC22m, ST-FMC46, ST-FMC46m, ST-CRC26, ST-CRC26m, ST-CRC32, and ST-CRC32m with the Rapid Annotation using Subsystem Technology (RAST) server identified multidrug resistance-conferring efflux pump genes *mdsB*, *mdtK*, *acrA*, and *sdiA* in all of the isolates (Table 6). Similarly, TET and sulfonamide resistance genes, *tetA* and *sul2*, were also present in all the isolates from conventional retailers.

Alterations of *S. Typhimurium* antibiotic resistance in soil environment. The acquisition/loss of antibiotic resistance in *S. Typhimurium* isolates ST-CRC26 and ST-FMC46 was investigated in soil environments under a biosafety level 2 (BSL-2) greenhouse facility, and an alteration of TET resistance was observed (Table 7). In both conventional and organic soil environments, isolate ST-CRC26 failed to lose TET resis-

TABLE 4 Gradual antibiotic resilience in *S. Typhimurium* isolates over 12 passages

Passage	Tetracycline MIC ($\mu\text{g/ml}$)		Sulfamethoxazole-trimethoprim MIC ($\mu\text{g/ml}$)	
	FMC22	FMC46	FMC22	FMC46
Original	4	4	19/1	19/1
1st	4	4	19/1	19/1
2nd	4	4	19/1	19/1
3rd	4	4	19/1	19/1
4th	8	8	19/1	19/1
5th	8	8	38/2	38/2
6th	8	8	38/2	38/2
7th	16	16	38/2	38/2
8th	16	16	38/2	38/2
9th	16	16	38/2	38/2
10th	32	32	38/2	38/2
11th	32	32	76/4	76/4
12th	32	64	76/4	76/4

TABLE 5 List of *S. Typhimurium* isolates and their genomic information

Isolate ^a	Draft genome length (bp)	No. of contigs	Coverage (×) ^b	N ₅₀ (bp)	Plasmid	SRA accession no.
ST-O340	4,896,263	85	334.28	224,483	A ^c	SRR5340795
ST-O335	4,897,698	87	211.50	224,465	A	SRR5340794
ST-O332	4,897,999	97	185.55	224,085	A	SRR5340793
ST-O292	4,898,827	92	218.81	247,396	A	SRR5340792
ST-O235	4,900,584	96	226.13	247,079	A	SRR5340791
ST-O231	4,897,200	90	272.10	301,394	A	SRR5340790
ST-O147	4,899,383	97	234.63	247,079	A	SRR5340789
ST-O135	4,897,940	98	280.49	301,394	A	SRR5340788
ST-O130	4,899,330	90	294.09	301,394	A	SRR5340787
ST-O124	4,895,924	89	237.82	301,394	A	SRR5340786
ST-O115	4,896,552	88	466.80	301,394	A	SRR5340785
ST-O105	4,898,745	89	235.75	223,861	A	SRR5340784
ST-O103	4,897,896	89	358.07	301,394	A	SRR5340783
ST-O102	4,896,521	83	236.67	225,432	A	SRR5340782
ST-O65	4,904,722	96	184.55	223,976	A	SRR5340781
ST-FMC333	4,897,765	80	224.80	301,394	A	SRR5340780
ST-FMC78	4,899,174	98	253.07	301,394	A	SRR5340779
ST-FMC77	4,896,928	87	138.26	301,394	A	SRR5340778
ST-FMC75	4,897,929	95	218.21	301,210	A	SRR5340777
ST-FMC74	4,896,614	97	231.70	224,404	A	SRR5340776
ST-FMC71	4,896,879	85	130.61	301,394	A	SRR5340775
ST-FMC70	4,895,993	84	262.49	301,394	A	SRR5340774
ST-FMC69	4,897,859	84	260.07	247,477	A	SRR5340773
ST-FMC68	4,897,555	92	265.08	247,096	A	SRR5340772
ST-FMC67	4,897,601	99	247.50	178,287	A	SRR5340771
ST-FMC65	4,898,898	92	177.14	301,394	A	SRR5340770
ST-FMC64	4,899,081	96	198.91	246,879	A	SRR5340769
ST-FMC63	4,899,787	102	153.67	247,869	A	SRR5340768
ST-FMC61	4,898,352	94	202.66	247,079	A	SRR5340767
ST-FMC59	4,897,860	93	284.11	301,394	A	SRR5340766
ST-FMC58	4,898,554	103	183.55	246,879	A	SRR5340765
ST-FMC57	4,895,754	84	239.10	301,394	A	SRR5340764
ST-FMC56	4,898,766	90	285.94	246,879	A	SRR5340763
ST-FMC51	4,897,186	87	285.17	225,553	A	SRR5340762
ST-FMC47	4,897,330	88	230.24	301,394	A	SRR5340761
ST-FMC46	4,897,764	84	218.10	301,394	A	SRR5340760
ST-FMC45	4,898,904	93	246.49	247,096	A	SRR5340759
ST-FMC44	4,898,789	99	188.28	224,012	A	SRR5340758
ST-FMC43	4,896,545	90	132.52	301,394	A	SRR5340757
ST-FMC42	4,897,826	93	190.90	247,079	A	SRR5340756
ST-FMC40	4,898,599	106	179.32	246,879	A	SRR5340755
ST-FMC34	4,898,862	98	243.88	296,746	A	SRR5340754
ST-FMC31	4,900,808	103	158.35	247,079	A	SRR5340753
ST-FMC28	4,898,294	86	265.12	301,394	A	SRR5340752
ST-FMC27	4,897,893	97	220.03	301,394	A	SRR5340751
ST-FMC24	4,895,878	89	217.42	301,394	A	SRR5340750
ST-FMC23	4,897,529	94	264.76	223,976	A	SRR5340749
ST-FMC22	4,897,495	87	253.85	225,432	A	SRR5340748
ST-FMC20	4,897,325	100	148.25	301,394	A	SRR5340747
ST-FMC14	4,898,908	111	105.89	246,576	A	SRR5340746
ST-FM76	4,898,908	104	130.47	224,270	A	SRR5340745
ST-CRC32	4,988,203	108	147.88	246,785	B ^d	SRR5340744
ST-CRC31	4,944,634	108	148.20	283,521	B	SRR5340743
ST-CRC29	4,948,215	150	143.24	208,466	B	SRR5340742
ST-CRC26	4,944,228	115	183.55	185,300	B	SRR5340741
ST-C32	5,001,673	159	171.41	229,563	B	SRR5340739
ST-LT2	4,897,621	104	99.02	301,394	A	SRR5340738
ST-21B	5,009,502	154	162.30	235,881	B	SRR5340737

^aAssembled draft genomes were analyzed with the tools available from the Center for Genomic Epidemiology web service, namely, ResFinder-2.1, KmerFinder-2.1, ContigAnalyzer-1.0, PlasmidFinder-1.2, MLST-1.6, pMLST-1.4, PathogenFinder-1.1, SeqSero-1.1, SalmonellaTypeFinder-1.2, and PathogenFinder-1.1.

^bCoverage per base pair was calculated using BMap (<https://sourceforge.net/projects/bbmap/>) and expressed as the mean. Assembled contigs were set as a reference, and trimmed pair end reads were mapped.

^cA, IncFIB(S) and IncFII(S).

^dB, IncA/C2 and ColRNAI.

FIG 1 SNP-based phylogenetic tree of *S. Typhimurium* isolates and selected *S. Typhimurium* genome sequences from GenBank. SNP detection and phylogenetic inference were performed by the CSI Phylogeny webserver; branch lengths correspond to numbers of nucleotide substitutions per site. Isolates from conventional retail chickens clustered with selected pathogenic *S. Typhimurium* strains (lineage 1) whereas most of the organic retailer and farmers' market chicken isolates clustered separately in lineages 3 and 4.

TABLE 6 Genes involved in antibiotic resistance in *S. Typhimurium* isolates

Function(s) ^a	Gene ^b	Presence (+) or absence (–) of gene in isolate:							
		ST-CRC26	ST-CRC26m	ST-CRC32	ST-CRC32m	ST-FMC22	ST-FMC22m	ST-FMC46	ST-FMC46m
Multidrug efflux	<i>tolC</i>					+	+	+	+
Multidrug efflux	<i>mdsB</i>	+	+	+	+	+	+	+	+
Multidrug efflux	<i>mdtK</i>	+	+	+	+	+	+	+	+
Multidrug efflux	<i>acrA</i>	+	+	+	+	+	+	+	+
Multidrug efflux	<i>sdiA</i>	+	+	+	+	+	+	+	+
Sulfonamide	<i>sul2</i>	+	+	+	+	–	–	–	–
Tetracycline	<i>tetA</i>	+	+	+	+	–	–	–	–
Resistance to antibiotics and toxins	<i>vapB</i>	+	–	+	+	+	+	+	+
Resistance to antibiotics and toxins	Membrane-bound lysozyme inhibitor of c-type lysozyme	+	–	–	–	+	+	+	+
Restriction modification	Type III restriction-modification system DNA endonuclease <i>res</i>	–	–	+	–	–	–	–	–
Cofactors	Dihydrolipoamide dehydrogenase	+	+	–	–	–	–	–	+

^aGene activity.^bBoth antibiotic resistance and functional genes.

tance, whereas isolate ST-FMC46 in both types of soil environment gradually acquired TET resistance, with higher MICs, over periods of time (Table 7).

More specifically, 100% of ST-CRC26 (12 of 12) isolates maintained their resistance potential against TET at a MIC of 64 $\mu\text{g/ml}$ during the first month in a TET-free soil

TABLE 7 Tetracycline resistance profiles of ST-FMC46 and ST-CRC26 in soil environment

Soil no. ^a	Strain ^b (tetracycline in $\mu\text{g/ml}$)	Isolate	MIC ($\mu\text{g/ml}$) at:				
			0 days	14 days	28 days	3 mo	6 mo
1	ST-FMC46 (4)	A5	4	32	64	128	NA ^c
		B3	4	4	64	128	128
		D1	4	4	4	16	64
		F1	4	4	4	128	128
		F3	4	4	4	128	NA
		F5	4	4	4	128	NA
2	ST-FMC46 (4)	A6	4	4	64	128	128
		D4	4	4	64	128	NA
		E1	4	32	128	128	128
		E3	4	4	4	128	128
		E7	4	32	128	128	NA
		F2	4	4	4	128	128
1	ST-CRC26 (128)	A3	64	64	64	128	NA
		A7	64	64	64	64	128
		A8	64	64	64	64	64
		B7	64	64	64	64	64
		C2	64	64	64	128	128
		D5	64	64	64	64	NA
2	ST-CRC26 (128)	B1	64	64	64	64	64
		B2	64	64	64	128	128
		C3	64	64	64	128	128
		C6	64	64	64	128	128
		F4	64	64	64	128	NA
		F8	64	64	64	64	128

^aSoil 1 was collected from a conventional farm; soil 2 was collected from an organic farm.^bST-FMC46, farmer's market chicken *S. Typhimurium* isolates susceptible to tetracycline. ST-CRC26, conventional retailer chicken *S. Typhimurium* isolates resistant to tetracycline. The resistance breakpoint of tetracycline is 32 $\mu\text{g/ml}$.^cNA, not applicable; *S. Typhimurium* isolates could not be recovered from soil samples.

TABLE 8 Differential expression of genes involved in antibiotic resistance in *S. Typhimurium* isolates

<i>Salmonella</i> isolate	Antibiotic resistance-related gene expression (fold) for:														
	<i>sdhA</i>	<i>tetA</i>	<i>sul2</i>	GeneA ^a	GeneB ^b	GeneC ^c	<i>vapB</i>	<i>mdfA</i>	<i>emrA</i>	<i>tolC</i>	<i>acrA</i>	<i>mdtA</i>	<i>mdsB</i>	<i>mdtK</i>	<i>macA</i>
ST-FMC46 ^d	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ST-FMC46m	0.35	0.18	0.15	0.91	0.96	0.40	2.32	1.25	0.55	1.40	1.54	0.39	0.45	0.83	1.66
A6	0.52	1,310.60	1,692.78	0.67	2.20	0.04	4.54	1.92	0.51	1.21	0.73	0.62	0.62	1.52	1.41
B3	0.52	3,296.49	13,375.23	1.50	1.71	0.31	1.00	0.44	0.37	2.06	2.20	1.84	1.22	0.91	1.15
E1	0.32	803.75	1,348.70	0.45	1.59	0.02	2.24	1.29	0.34	1.00	0.53	0.33	0.41	1.07	0.93
F1	0.44	653.92	42.45	0.64	2.08		2.31	0.47	0.41	0.78	0.89	0.28	0.51	1.14	1.38
ST-CRC26 ^e	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
ST-CRC26m	0.74	1.92	2.46	1.19	0.37	0.40	0.34	0.40	0.88	1.67	1.53	0.70	1.08	0.48	1.22
A8	0.64	0.36	0.26	1.00	0.91		0.53	1.11	0.55	1.18	0.82	0.97	1.21	0.77	0.85
B1	1.24	1.60	1.83	1.42	1.14	1.93	1.17	1.19	1.17	1.11	1.47	1.89	2.06	1.21	2.20
B7	0.78	1.03	5.31	1.29	0.27		0.36	0.26	0.44	1.09	1.79	0.81	0.78	0.65	0.78
F8	1.29	0.96	3.86	1.52	1.03	1.01	0.88	1.38	1.01	1.23	1.59	2.43	2.27	0.89	1.33

^aGeneA, membrane-bound lysozyme inhibitor of c-type lysozyme gene.

^bGeneB, type III restriction-modification system DNA endonuclease *res*.

^cGeneC, dihydrolipoamide dehydrogenase gene.

^dGene expression levels of *Salmonella* isolates FMC46m, E1, A6, B3, and F1 were normalized against the expression level of FMC46.

^eGene expression levels of *Salmonella* isolates CRC26m, A8, F8, B1, and B7 were normalized against the expression level of CRC26.

environment. After the second month, one of the recovered ST-CRC26 isolates raised its MIC against TET to 128 $\mu\text{g/ml}$, and 5 other isolates of ST-CRC26 recovered from soil samples also reached a MIC of 128 $\mu\text{g/ml}$ against TET after the third month. Eventually, at least 2 ST-CRC26 isolates recovered from conventional farm soil and 4 isolates recovered from organic farm soil (in total, at least 6 out of 12 isolates) stabilized their MICs against TET at 128 $\mu\text{g/ml}$, which is double the TET resistance breaking point.

On the other hand, with 4 mg TET per kg soil supplement, only 1 out of 6 ST-FMC46 (16.6%) isolates recovered from conventional soil culture environment and 2 out of 6 ST-FMC46 (33.3%) isolates recovered from organic soil acquired TET resistance (MIC = 32 $\mu\text{g/ml}$) within 2 weeks. Additionally, another 3 ST-FMC46 isolates from soil samples reached the TET resistance breakpoint after 1 month. After 3 months, 11 out of 12 (91.6%) recovered isolates had MIC values of 128 $\mu\text{g/ml}$. Within 6 months in the soil environments, all of the recovered cultivable ST-FMC46 isolates were TET resistant.

However, no significant alteration in SXT resistance was observed within 6 months of sublethal SXT supplement or non-antibiotic-resistance induction (data not shown). All 12 susceptible ST-FMC46 isolates recovered from soil environments retained their SXT MIC at 19/1 $\mu\text{g/ml}$ after 14 days and after 1, 2, 3, and 6 months. Similarly, all 12 recovered resistant ST-CRC26 isolates retained their SXT MIC at 76/4 $\mu\text{g/ml}$ after 14 days and after 1, 2, 3, and 6 months. In other words, none of the 12 recovered ST-FMC46 isolates developed SXT resistance in a soil environment, and all of the 12 ST-CRC26 isolates recovered from soil environments maintained their SXT resistance.

Differential expression of genes involved in antibiotic resistance. Differential expression of genes involved in antibiotic resistance in *S. Typhimurium* isolates that were artificially inoculated into soil under antibiotic pressure was determined with quantitative real-time PCR (qRT-PCR) and is presented in Table 8. Two *S. Typhimurium* isolates, ST-FMC46 (TET sensitive) and ST-CRC26 (TET resistant), were inoculated in the presence or absence of TET, respectively. We observed, with TET pressure, that expression of the *tetA* gene was upregulated by 803-, 1,310-, 3,296-, and 653-fold in isolates E1, A6, B3, and F1, respectively, compared to that in the wild-type control isolate, ST-FMC46. Collateral damage of TET in soil was noticed, and significant upregulation of *sul2* was also observed in these isolates (by 1,348-, 1,692-, 13,375-, and 42-fold in E1, A6, B3, and F1, respectively) compared to expression in ST-FMC46. Expression levels of *vapB* in E1, F6, and A1 were upregulated by 2.24-, 4.54-, and 2.31-fold, respectively, compared to that in the wild-type control isolate, ST-FMC46. On the other hand, antibiotic pressure was associated with downregulation of the dihydrolipoamide dehydrogenase

and *emrA* genes in these isolates, while expression of *res*, *mdfA*, *tolC*, *acrA*, *mdtK*, and *macA* remained unaltered. The presence of TET also resulted in the downregulation of *sdiA* in all of the isolates compared to expression in ST-FMC46. In the absence of TET, expression of *tetA* and *vapB* genes was downregulated in isolates A8, F8, and B7 compared to that in the wild-type control, ST-CRC26. Alterations in the expression levels of *tetA* in the absence or presence of TET were highly noticeable, i.e., addition of TET resulted in a more than 40-fold alteration of expression, but its absence resulted in a less than 2-fold alteration.

DISCUSSION

Risks of *Salmonella* prevalence and drug resistance in farm systems. Our previous study demonstrated the potential for *Salmonella* risks with both conventional and mixed crop-livestock (MCL) farm systems. A higher overall prevalence of *Salmonella* contamination was detected in the MCL system, whereas multiantibiotic resistance in *Salmonella* was found predominantly in conventional farms, where farmers are allowed to use synthetic antibiotics, and in their related postharvest products in retail stores (25). In this study, we further investigated phylogenetically inferred relatedness, genomic characteristics, and the alteration of antibiotic resistance-related genes under environmental pressures of the designated *S. Typhimurium* isolates. Surprisingly, we found that conventional farm *S. Typhimurium* isolates were closely related to pathogenic *S. Typhimurium* strain 140285 (GenBank accession number [CP001363](#)), while MCL or organic retailer *S. Typhimurium* isolates were highly genetically similar to the isolates from nonpathogenic *S. Typhimurium* strain ST-LT2 (GenBank accession number [NC_003197](#)). Additionally, previous epidemiological studies suggested that patients infected with multidrug-resistant *Salmonella* strains commonly suffered prolonged illnesses of greater severity than patients infected with susceptible *Salmonella*, suggesting a possible association between the antibiotic resistance and the virulence/pathogenicity of *Salmonella* strains (28–30), which also supported our findings.

Rapid acquisition of antibiotic resistance in susceptible *Salmonella* isolates. Several groups of researchers have previously claimed that the rise in resistance came not only from clinical/veterinary therapeutic uses but also from subtherapeutic application of antibiotics in farm animals, as well as chemical contamination under certain environmental conditions (31). Through supplementation of subtherapeutic doses of antibiotics in natural soil environments, we identified antibiotic resistance gene accumulation in several *S. Typhimurium* isolates at different time points. Under short-term (less than 11 *in vitro* passages or 14 days of soil cultivation) antibiotic pressure, pansusceptible *S. Typhimurium* isolates gradually developed TET resistance. However, multidrug-resistant *S. Typhimurium* isolates barely lost or sacrificed their drug resistance, even after 12 passages *in vitro* or 6 months in soil environments in the absence of antibiotics. On the other hand, we also observed continuous expansion of TET resistance among several pansusceptible *S. Typhimurium* isolates in an unadulterated soil environment (data not shown). Generally, bacteriophages and mobile genetic elements serve as the antibiotic resistome in soil environments (32). Consequently, pathogenic bacteria like *Salmonella enterica* could potentially acquire resistance genes against specific antibiotics through horizontal gene transfer from this microbial resistome, which serves as the resistance gene reservoir.

The efflux pump system encoded by *tet* genes (e.g., *tetA*) serves as the main TET resistance mechanism in various bacterial pathogens, including *Salmonella* (33). Accordingly, in our study, we found a substantial association between TET pressure and the upregulated expression level of *tetA* in *S. Typhimurium*. Similarly, in the absence of TET, we also observed significant downregulation of *tetA* in *Salmonella*. Additionally, the upregulation under sublethal TET induction of *vap* genes in *Salmonella*, which encode small secreted virulence-associated proteins in bacterial pathogens, further supports our SNP-based phylogenetic inference results. In addition to proximity-dependent horizontal gene transfer, extra environmental factors—specifically, antibiotic and/or chemical pressure—could play a crucial role in the transfer of antibiotic

resistance genes among members of the bacterial/microbial community. For example, an acidic or alkaline incubation environment was capable of stimulating horizontal transfer of TET resistance genes located on plasmids (34). In our study, environmental factors (e.g., temperature, air condition, humidity, pH, and time of cultivation) influence the metabolic rate and growth productivity of soil bacteria, potentially decreasing the cultivability of *S. Typhimurium* after 6 months (35). Furthermore, the existence of a TET resistome in natural soil environments might further explain the difficulty in losing TET resistance observed in resistant strains, even in the absence of antibiotic pressure.

Unexpectedly, the induction of SXT declined in building up resistance in pansusceptible *S. Typhimurium* isolates. This might be due to the intricacy of the mechanism of action of sulfonamides, which operates by interfering with bacterial folate synthesis (36). In such cases, the synergistic effects in combination with supplemented sulfamethoxazole and trimethoprim might play an important role (37). Although several sulfonamide resistance genes were recognized in soils fertilized with animal manure or organic matter (38), the buildup of their resistance in *Salmonella* remains continuous and moderate, which may generally require years of interventions (39). Correspondingly, stagnant progression or the lack of sulfonamide resistance genetic elements might explain the failure in this study of SXT resistance development via sublethal dose of antibiotic induction either within 12 passages of *in vitro* culture or after months of natural soil incubation. However, similarly to the TET-resistant isolates, the original SXT-resistant ST-CRC isolate did not show decreased resistance toward sulfonamides. Indeed, we expected that sulfonamide resistance could be even more rigid than TET resistance, since most bacteria have to alter their metabolic pathway for nucleic acid and folic acid synthesis, which is hardly reversible even in the absence of sulfonamide induction (39, 40).

Association of drug resistance and relatedness with virulence. As increases in antibiotic resistance and virulence properties were usually observed concurrently (41, 42), their genetic connection and global genetic regulation are inevitably important for this simultaneous phenomenon (43). Regulation of antibiotic resistance and virulence genes is intertwined and highly complex, being influenced by multiple environmental factors, quorum sensing, and two-component systems that are believed to act as the connection in coincident upregulation (43, 44). In this study, we demonstrated the rapid acquisition of drug resistance, as well as the upregulation of antibiotic resistance-related genes in *S. Typhimurium* isolates, and the close relatedness between drug-resistant and pathogenic *S. Typhimurium* strains. However, further study of the mechanisms of their coordinate regulators and coregulation system networks is necessary to uncover the key interaction/connection between antibiotic resistance and virulence, which might induce novel insights into drug invention against persistent bacterial infections.

Conclusions. This study demonstrated alterations of antibiotic resistance in *S. Typhimurium* isolates from conventional farm systems under environmental pressures. Although multiantibiotic-resistant *S. Typhimurium* isolates exhibited barely reduced resistance, the pansusceptible isolates managed to develop TET resistance quickly in both *in vitro* and soil environments. Additionally, the relative bacterial genetic alterations withstanding the rapid physical buildup of antibiotic resistance in *S. Typhimurium* indicated their expeditious acquisition of resistance genes from environmental reservoirs. Tracking and understanding the phenotypical transitions of antibiotic resistance in the microbial ecosystem will offer us more specific and directed strategies in combating dissemination of antibiotic resistance in *Salmonella* and minimizing its virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A total of 54 *S. Typhimurium* isolates were used and analyzed in this study. Sources, identification, and characterization of isolates were reported in our previous article (25). Briefly, all samples were collected in either organic or conventional poultry farms and retail markets, including organic and conventional markets and local farmers' markets in Maryland (MD) and the District of Columbia (DC) metropolitan areas. The sources and distribution of these isolates

are listed in Table 1. The antibiotic resistance profiles of the 54 *S. Typhimurium* isolates are summarized in Table 2. All of the *S. Typhimurium* isolates were recovered from stock culture (Luria-Bertani [LB] broth with 20% glycerol stored in a -80°C freezer) on LB agar overnight (18 h) at 37°C under aerobic conditions. The recovered isolates were consecutively subcultured three times on LB agar overnight at 37°C under aerobic conditions for further investigation.

In vitro investigation of *S. Typhimurium* antibiotic resistance shifts. Two *S. Typhimurium* isolates with TET and SXT resistance, ST-CRC26 and ST-CRC32, and two pansusceptible isolates, ST-FMC22 and ST-FMC46, were used for this phase of the study. The antibiotic-resistant isolates were used for *in vitro* examination of their antibiotic resistance shifts with and without antibiotic pressure in a nutritious environment. ST-CRC26 and ST-CRC32 were consecutively passaged 12 times (24 h per passage) in LB broth supplemented with 10% sheep blood and then incubated for 24 h at 37°C under aerobic condition.

Approximately 10^6 CFU/ml of ST-FMC22 and ST-FMC46 isolates in phosphate-buffered saline (PBS), measured at an optical density at 600 nm of around 0.01 with a LAMBDA BIO/BIO+ spectrophotometer (PerkinElmer, Beaconsfield, UK), were sequentially cultured in LB broth containing gradually increasing concentrations of antibiotic, as follows: TET from 2 to 128 $\mu\text{g/ml}$ and SXT from 9.5/0.5 to 152/8 $\mu\text{g/ml}$. The surviving bacteria were subcultured into double-concentrated, fresh, antibiotic-containing LB broth for 24 h at 37°C under aerobic conditions. After final passage, the isolated strains were labeled ST-CRC26m, ST-CRC32m, ST-FMC22m, and ST-FMC46m and stored in -80°C glycerol-containing stock culture for future use.

Whole-genome sequencing of *S. Typhimurium* isolates. The *S. Typhimurium* isolates were cultured on LB agar, and a single colony was transferred to 10 ml of LB broth and incubated aerobically for 24 h at 37°C . After incubation, the liquid cultures were centrifuged and decanted, and the pellets were processed for DNA extraction using a Qiagen DNeasy kit (Qiagen, Valencia, CA). Nextera XT libraries were prepared separately for each of the samples and pooled into equimolar concentrations, following the instructions of the manufacturer (Illumina, San Diego, CA). A high-output flow cell was used for paired-end sequencing (2×151 bp) with an Illumina NextSeq 500 sequencing platform. Data were demultiplexed and trimmed to remove adaptor sequences using the BCL2FastQ program, and PhiX reads were removed using DeconSeq (45). Reads were further cleaned using Trimmomatic V 0.36 (leading, 20; trailing, 20; sliding, 4:20; minimum length [minlen], 36) (46). Only paired data were further analyzed and assembled using SPAdes 3.6.2 with paired-end libraries ($-pe$) and pipeline options ($-careful$) (47). Assembled contigs were analyzed with the following Center for Genomic Epidemiology pipelines: ResFinder-2.1, KmerFinder-2.1, ContigAnalyzer-1.0, MLST-1.6, PlasmidFinder-1.6, and *Salmonella*TypeFinder-1.2. Single nucleotide polymorphisms (SNPs) were identified using CSI Phylogeny-1.4, available from the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org>) (48), where the paired-end reads from each isolate were aligned against the reference genome, ST-LT2 (ATCC 700720). The qualified SNPs were designated based on the following criteria: (i) a minimum coverage of $10\times$; (ii) a minimum of 10 bp between SNPs; (iii) a minimum quality score of 30; (iv) a minimum read mapping quality of 25; and (v) a minimum Z-score of 1.96 with the altered FastTree option activated for more accuracy. The chromosome of ST-LT2 was selected as the reference genome for SNP identification. To infer the phylogeny of the *S. Typhimurium* isolates, the SNP matrices in Newick format were imported to FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>). Assembled contigs of *S. Typhimurium* isolates ST-FMC22, ST-FMC22m, ST-FMC46, ST-FMC46m, ST-CRC26, ST-CRC26m, ST-CRC32, and ST-CRC32m were further annotated with the Rapid Annotation using Subsystem Technology (RAST) server (<http://rast.nmpdr.org/rast.cgi>).

Soil environment simulation of *S. Typhimurium* antibiotic resistance development. To verify our *in vitro* finding of *Salmonella* antibiotic resistance shifts under antibiotic pressure or absence of antibiotic, the same study was performed in a soil environment. For this study, natural soil samples were collected from both conventional and organic farms and designated soil 1 and soil 2, respectively. Both soil samples were stored in plastic buckets at room temperature and then air-dried by spreading on separate aluminum trays in a growth chamber for 3 days. The dried soil samples were sealed and stored in separate sterilized plastic bags.

Antibiotic liquid stock solutions contained 4 mg TET per ml distilled water (dH_2O) and 19/1 mg SXT per ml dH_2O . An aliquot of 1 ml of both antibiotic stocks was sprayed and mixed well in 1 kg soil sample labeled as Ab^+ with antibiotic supplement in a final sublethal concentration of 4 mg TET per kg soil and 19/1 mg SXT per kg soil. The rest of the soil was supplemented with the same amount of dH_2O , labeled as Ab^- for control. An aliquot of 2 ml of either ST-CRC26 or ST-FMC46 bacterial suspension stock (10^8 CFU per ml PBS) was inoculated and mixed well into 1 kg soil sample labeled separately as ST-CRC26 or ST-FMC46 group, for a final bacterial concentration of 10^7 CFU per 50 g soil. Then, each 250-g supplemented soil sample was weighed and filled up in labeled matching clusters following the random predesigned experimental soil panel shown in Fig. 2. Each antibiotic supplementation was replicated a total of 6 times (6 clusters), and each cluster was subdivided into 5 cells for 5 time period samplings for further processing and antibiotic resistance tests as per standard procedure. The supplemented soil cells were cultured in a growth chamber (Thermo Fisher Scientific Inc., Waltham, MA) and gently irrigated with tap water every 2 days.

Soil sampling, processing, and enrichment for recovering *S. Typhimurium* isolates. Soil samples containing *S. Typhimurium* isolates were collected and processed on day 0 and at 14 days, 28 days, and 2 months for the short-term investigation, plus at 3 months and 6 months for the long-term investigation. Each sample was processed separately, following "*Salmonella*" in the Bacteriological Analytical Manual (49). Briefly, 1 g soil sample from each soil panel cell was weighed, collected in a sterilized Eppendorf tube, and mixed into 9 ml of buffered peptone water for overnight incubation at 37°C under

FIG 2 *S. Typhimurium* inoculation panel in soil environment. Soil samples were collected from two types of farms, with soil 1 as conventional farm soil and soil 2 as organic farm soil. ST-CRC26 or ST-FMC46 isolate was applied for inoculation. Ab⁺ indicates antibiotic supplement in a final sublethal concentration of 4 mg per kg soil for tetracycline and 19/1 mg per kg soil for SXT. Ab⁻ indicates no antibiotic supplement.

aerobic conditions, followed by Rappaport-Vassiliadis medium and tetrathionate broth overnight sub-culture at 37°C under aerobic conditions. The final enriched bacteria in tetrathionate broth were plated on xylose-lysine-tergitol 4 (XLT-4) agar at 37°C under aerobic conditions for *Salmonella* isolation. Triple colonies from each sample were subcultured on Mueller-Hinton (MH) agar overnight at 37°C under aerobic conditions for both -80°C glycerol stock and further MIC determination.

Determination of TET and SXT resistance of *S. Typhimurium* isolates recovered from soil samples. TET and SXT resistance of *Salmonella* isolates was tested with a standard agar dilution method according to the Clinical and Laboratory Standards Institute (50). In total, 5 different concentrations (2-fold serial dilution) of antimicrobial solutions were mixed into freshly prepared MH agar. The isolated *Salmonella* colonies were picked up using sterile inoculation loops and suspended in PBS for McFarland standard (0.5) adjustment. The standardized bacterial suspension was diluted by 10-fold with PBS, and then 2 µl of each bacterial suspension containing approximately 10⁵ CFU was spotted on antibiotic-containing MH agar for overnight incubation at 37°C under aerobic conditions. The MIC of each antibiotic was recorded as the lowest concentration that completely inhibited the visible growth of the *Salmonella* isolate.

Quantitative RT-PCR assay. All isolates of ST-FMC22, ST-FMC22m, ST-FMC46, ST-FMC46m, ST-CRC26, ST-CRC26m, ST-CRC32, and ST-CRC32m were cultured in LB broth at 37°C overnight. The bacterial cells were harvested; RNA extraction was carried out, followed by cDNA synthesis; and qRT-PCR was performed in an Eco RT-PCR system (Illumina, CA), according to the protocol previously described (51, 52). The PCR cycle was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 55°C for 15 s, and 72°C for 10 s. The custom-synthesized oligonucleotides (Erofin MWG Operon, Huntsville, AL) were used as primers to target conserved regions of *S. Typhimurium* (see Table S1 in the supplemental material). The relative transcriptional expression levels of target genes were estimated by the comparative fold change. The housekeeping gene was used as the reference gene for normalization of target gene expression. Quantitative RT-PCR assays were carried out in triplicate and the results were averaged.

Accession number(s). Accession numbers of draft genome contigs have been deposited in the NCBI Sequence Read Archive and are listed in Table 5.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01173-18>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

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