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PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Horizontal Dissemination of Antimicrobial Resistance Determinants in Multiple *Salmonella* Serotypes following Isolation from the Commercial Swine Operation Environment after Manure Application

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ABSTRACT The aim of this study was to characterize the plasmids carrying antimicrobial resistance (AMR) determinants in multiple *Salmonella* serotypes recovered from the commercial swine farm environment after manure application on land. Manure and soil samples were collected on day 0 before and after manure application on six farms in North Carolina, and sequential soil samples were recollected on days 7, 14, and 21 from the same plots. All environmental samples were processed for *Salmonella*, and their plasmid contents were further characterized. A total of 14 isolates including *Salmonella enterica* serotypes Johannesburg ($n = 2$), Ohio ($n = 2$), Rissen ($n = 1$), Typhimurium var5− ($n = 5$), Worthington ($n = 3$), and 4,12:i:− ($n = 1$), representing different farms, were selected for plasmid analysis. Antimicrobial susceptibility testing was done by broth microdilution against a panel of 14 antimicrobials on the 14 confirmed transconjugants after conjugation assays. The plasmids were isolated by modified alkaline lysis, and PCRs were performed on purified plasmid DNA to identify the AMR determinants and the plasmid replicon types. The plasmids were sequenced for further analysis and to compare profiles and create phylogenetic trees. A class 1 integron with an ANT(2″)-Ia-*aadA2* cassette was detected in the 50-kb IncN plasmids identified in *S.* Worthington isolates. We identified 100-kb and 90-kb IncI1 plasmids in *S.* Johannesburg and *S.* Rissen isolates carrying the bla_{CMY-2} and *tet*(A) genes, respectively. An identical 95-kb IncF plasmid was widely disseminated among the different serotypes and across different farms. Our study provides evidence on the importance of horizontal dissemination of resistance determinants through plasmids of multiple *Salmonella* serotypes distributed across commercial swine farms after manure application.

IMPORTANCE The horizontal gene transfer of antimicrobial resistance (AMR) determinants located on plasmids is considered to be the main reason for the rapid proliferation and spread of drug resistance. The deposition of manure generated in swine production systems into the environment is identified as a potential source of AMR dissemination. In this study, AMR gene-carrying plasmids were detected in multiple *Salmonella* serotypes across different commercial swine farms in North Carolina. The plasmid profiles were characterized based on *Salmonella* serotype donors and incompatibility (Inc) groups. We found that different Inc plasmids showed evidence of AMR gene transfer in multiple *Salmonella* serotypes. We detected an identical 95-kb plasmid that was widely distributed across swine farms in North Carolina. These conjugable resistance plasmids were able to persist on land after swine ma-

Received 9 July 2017 **Accepted** 9 August 2017

Accepted manuscript posted online 11 August 2017

Citation Pornsukarom S, Thakur S. 2017. Horizontal dissemination of antimicrobial resistance determinants in multiple *Salmonella* serotypes following isolation from the commercial swine operation environment after manure application. Appl Environ Microbiol 83:e01503-17. https://doi.org/10.1128/AEM.01503-17.

Editor Donald W. Schaffner, Rutgers, The State University of New Jersey

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nure application. Our study provides strong evidence of AMR determinant dissemination present in plasmids of multiple *Salmonella* serotypes in the environment after manure application.

KEYWORDS *Salmonella*, plasmid, antimicrobial resistance, horizontal gene transfer, environment, environmental microbiology, swine farm

The emergence of antimicrobial resistance (AMR) in bacterial pathogens has threatened the sustainability of an effective global public health response to infectious diseases (1, 2). There are major gaps in our understanding of AMR transmission within agricultural sites and the potential impacts on humans, animals, and the environment due to a lack of studies conducted on actual commercial food animal farms (3–5). A number of studies have documented the abundance of AMR pathogens associated with livestock production due to the intensive use of antimicrobials in animal husbandry practices for therapeutic and nontherapeutic purposes (6–9). However, there is limited knowledge about the effect of manure application on the spread of AMR pathogens and AMR genes by means of horizontal gene transfer (HGT), such as by plasmids, transposons, and integrons, in the environment (4, 10, 11). Exposure of bacterial pathogens to antimicrobials in the environment increases the evolution of resistance and has an influence on the abundance, distribution, and transfer of AMR genes into different bacterial species (9, 12). We recently reported the dissemination of AMR *Salmonella* isolates in manure from commercial swine farms that were able to persist on land for at least 21 days after manure application, and it was clearly observed that *Salmonella* bacteria were rarely present in the soil before the land application (13). Given the potential risk of disseminating AMR *Salmonella* bacteria into the environment during manure application, we further characterized the plasmids that were detected in the multiple *Salmonella* serotypes isolated in our previous study.

The dissemination of undesirable AMR genes in Gram-negative pathogenic bacteria has been mainly regarded as the acquisition of multiple plasmid-located AMR genes by HGT (14, 15). Conjugation is considered the main mode of HGT of AMR genes among the *Enterobacteriaceae* family and helps to increase bacterial genetic diversity (16, 17). Plasmids conferring resistance have been identified as hindering antimicrobial therapy, including the use of extended-spectrum cephalosporins and fluoroquinolones, which are regarded as drugs of choice for bacterial infection in human clinical cases (14, 18, 19). Studies from several parts of the world have demonstrated the distribution of plasmids harboring extended-spectrum β-lactamase (ESBL) genes (bla_{CTX}, bla_{SHV}, bla_{CMY}, and bla_{TEM}) or *ampC* and plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, and *qnrS*) in *Escherichia coli* and *Salmonella* among animal, human, and environmental sources (16, 20–22). The presence of plasmid-mediated transfer of a recently identified mobile colistin resistance gene (*mcr-1*) is another example of the threat posed to public health (23, 24). The comparative analysis of *mcr-1*-containing plasmids maintained in the *Enterobacteriaceae* family revealed that they are disseminated in a broad host range, including human, animal, and food sources, and are now being reported from different countries worldwide (25–27). Plasmids that confer resistance in carbapenem-resistant *Enterobacteriaceae* (CRE) pose an urgent threat to public health with their global expansion (28, 29). Mollenkopf et al. (30) reported that the CRE carrying bla_{IMP-27} plasmids were recovered from the environment of a swine production area in the United States. The farm environment is considered a potential reservoir of AMR *Salmonella* strains that probably exchange AMR determinants with humans and animals by plasmid horizontal transfer (13, 22, 31, 32).

The objective of this study was to determine and characterize the resistance plasmid profiles isolated from multiple AMR *Salmonella* serotypes recovered in manure and environmental samples after land application of manure on commercial swine farms in North Carolina. To address this, we performed antimicrobial susceptibility testing (AST), plasmid replicon typing, conjugation assays, and plasmid sequencing to fully understand the role of these plasmids in transferring AMR determinants in the environment.

RESULTS

***Salmonella* serotypes and plasmid characterization.** A total of 14 different *Salmonella* serotypes isolated from commercial swine farms in North Carolina were selected to determine whether the AMR genes were located on transmissible plasmids. We also wanted to find out whether dissemination of AMR *Salmonella* bacteria through manure application assists in the transmission of genes via plasmids to other susceptible bacterial populations. *Salmonella* isolates collected from the swine farm environment after manure application were selected from each farm based on type of sample (lagoon and soil), sampling day, serotype, and resistance phenotype (Table 1). All 14 *Salmonella* donors harbored at least one large plasmid larger than 40 kb in size, and their plasmid profiles were dependent on farm origin and donor *Salmonella* serotype. PCR-based replicon typing (PBRT) revealed four plasmid replicons (FI, FII, I1, and N) among the 14 isolates carrying plasmids (Table 1). IncN plasmids ($n = 3$) of 50 kb in size were found in *Salmonella enterica* serotype Worthington isolates from both lagoon and soil samples in North Carolina farm 1 (NCF1). In NCF3, 100-kb IncI1 ($n = 2$) plasmids were isolated from *S. enterica* serotype Johannesburg while *S. enterica* serotype Typhimurium var5− was the predominant serotype at this farm and carried IncFII plasmids ($n = 4$) of 95 kb in size. Furthermore, IncFII plasmids were also found in *S.* Typhimurium var5− from NCF5 and 4,12:i:− from NCF6. A single *S. enterica* serotype Rissen isolate from a lagoon sample in NCF6 carried an IncI1 plasmid of 90 kb in size. The heterogeneous IncF group was the predominant replicon type detected in this study. Within the IncF group, we detected the subgroups FIA, FIB, FIC, FIIA, and F_{rep}, with IncFIC and F_{rep} being the most prevalent subgroups. The IncFI plasmid group found in 10 *Salmonella* isolates (Table 1) was determined to consist of small plasmids (less than 40 kb in size each). However, the plasmids identified in our study were represented by more than one replicon family in each isolate.

Antimicrobial resistance phenotypes. To determine the AMR phenotypes and MICs for all 14 nalidixic acid-resistant (NAL^r) *E. coli* confirmed transconjugants and the 14 AMR *Salmonella* donor isolates from the environmental source, we conducted antimicrobial susceptibility testing using broth microdilution. The results of transconjugant AST correlated with the AMR profiles and the MICs for the *Salmonella* donor isolates, confirming the successful transfer of plasmids from the donors to the recipient strains (Table 2). NAL^r was detected in all 14 transconjugants since the NAL^r *E. coli* JM109 strain was used as a recipient for plasmid transfer. Five out of 14 plasmids were considered multidrug resistant (MDR; resistant to more than three classes of antimicrobials) including plasmids pS6 (*S.* Worthington donor), pS9 and pS10 (*S.* Johannesburg donor), pS24 (*S. enterica* 4,12:i:− donor), and pS27 (*S.* Typhimurium var5− donor) (Table 1). The plasmid pS6 showed resistance to sulfisoxazole (FIS), gentamicin (GEN), streptomycin (STR), and tetracycline (TET), while plasmids pS7 and pS8 had the MDR pattern FIS-STR-TET. These three transconjugants were successfully transferred to the recipient *E. coli* from *S.* Worthington donors recovered from NCF1, but only transconjugant pS6 had a 100% AMR profile that matched that of the donor isolate. Two plasmids, pS9 and pS10, were isolated from transconjugants of *S.* Johannesburg on NCF3 representing identical MDR patterns, with resistance to ampicillin (AMP), amoxicillin-clavulanic acid (AUG2), ceftriaxone (AXO), and cefoxitin (FOX). However, the ceftiofur (XNL) resistance represented in *S.* Johannesburg isolates was not detected in the transconjugants (Table 2). The plasmid pS27 from *S.* Typhimurium var5− recovered from NCF5 showed resistance to AMP, chloramphenicol (CHL), FIS, STR, and TET. Plasmids pS12, pS13, pS14, and pS15 isolated from transconjugants of *S.* Typhimurium var5− on farm 3 had the resistance pattern AMP-FIS. The plasmid from NCF6, pS24 with the MDR pattern AMP-FIS-STR-TET, was isolated from an *S. enterica* 4,12:i:− transconjugant. Another plasmid from farm 6 (pS20) from *S.* Rissen was resistant to only TET. All the transconjugants with AMP resistance were selected on Luria-Bertani (LB) plates with AMP and NAL as the markers, while the rest of the transconjugants were selected on NAL and TET marker LB plates.

TABLE 1 Conjugative resistance plasmid content of 14 environmental isolates harboring AMR genes recovered from *Salmonella* donor isolates after manure application on commercial swine farms in North Carolina

Salmonella donor isolate			Plasmid					
Farm and source[a]	Day of sampling	Serotype	Name	Size (kb)	Inc group[b]	ST (Inc group)[c]	Resistance pattern (MIC [μg/ml])[d]	AMR gene(s)
NCF1								
Lagoon	0	Worthington	pS6	50	N (50 kb), FI	ST5 (N)	FIS (>256), GEN (16), STR (64), TET (>32)	*sul1*, *aadA2*, *tet*(A)
Soil	0	Worthington	pS7	50	N (50 kb), FI	ST5 (N)	FIS (>256), STR (64), TET (>32)	*sul1*, *aadA2*, *tet*(A)
Soil	7	Worthington	pS8	50	N (50 kb), FI	ST5 (N)	FIS (>256), STR (64), TET (>32)	*sul1*, *aadA2*, *tet*(A)
NCF3								
Lagoon	0	Johannesburg	pS9	100, 95	I1 (100 kb), FI (95 kb)	ST12 (I1)	AMP (>32), AUG2 (32/16), AXO (16), FOX(32)	bla_{CMY-2}
Soil	7	Johannesburg	pS10	95	I1, FI (95 kb)		AMP (>32), AUG2 (32/16), AXO (16), FOX (32)	bla_{CMY-2}
Lagoon	0	Typhimurium var5−	pS12	95	FII, FI		AMP (>32), FIS (>256)	*sul1*
Soil	7	Typhimurium var5−	pS13	95	FII, FI		AMP(>32), FIS(>256)	*sul1*
Soil	14	Typhimurium var5−	pS14	95	FII		AMP (>32), FIS (>256)	
Soil	21	Typhimurium var5−	pS15	95	FII		AMP (>32), FIS (>256)	
NCF6								
Lagoon	0	Rissen	pS20	90	I1 (90 kb), FI	ST155 (I1)	TET(>32)	*tet*(A), *tet*(B)
Soil	0	4,12:i:−	pS24	95	FII, FI		AMP (>32), FIS (>256), STR (>64), TET (>32)	bla_{TEM}, *sul2*, *aadA*
NCF5								
Lagoon	0	Typhimurium var5−	pS27	95	FII, FI		AMP (>32), CHL (>32), FIS (>256), STR (32), TET (32)	*sul1*, *aadA2*
Lagoon	0	Ohio	pS28	40	FI		TET (>32)	*tet*(A)
Soil	0	Ohio	pS29	40	FI		TET (>32)	*tet*(A)

[a]NCF, North Carolina farm.

[b]Incompatibility group based on PBRT (45).

[c]Sequence type (ST) based on pMLST (https://pubmlst.org/plasmid/) (41).

[d]Nalidixic acid (NAL) resistance was not detected in the plasmid isolated from the transconjugant. MIC ranges of the drugs are as follows: amoxicillin-clavulanic acid (AUG2), 1/0.5 to 32/16 μg/ml (breakpoint, ≥32/16 μg/ml); ampicillin (AMP), 1 to 32 μg/ml (breakpoint, ≥32 μg/ml); cefoxitin (FOX), 0.5 to 32 μg/ml (breakpoint, ≥32 μg/ml); ceftriaxone (AXO), 0.25 to 64 μg/ml (breakpoint, ≥4 μg/ml); chloramphenicol (CHL), 2 to 32 μg/ml (breakpoint, ≥32 μg/ml); gentamicin (GEN), 0.25 to 16 μg/ml (breakpoint, ≥16 μg/ml); streptomycin (STR), 32 to 64 μg/ml (breakpoint, ≥32 μg/ml); sulfisoxazole (FIS), 16 to 256 μg/ml (breakpoint, ≥512 μg/ml); and tetracycline (TET) 4 to 32 μg/ml (breakpoint, ≥16 μg/ml).

TABLE 2 Antimicrobial susceptibilities with MICs of AMR environmental *Salmonella* isolates and corresponding *E. coli* transconjugants

Salmonella isolate or transconjugant[a]	MIC (μg/ml)[b]													
	AMP	AUG2	AXO	AZI	CHL	CIP	FIS	FOX	GEN	NAL	STR	SXT	XNL	TET
S6	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**16**	2	**64**	<0.12/2.38	1	**>32**
TC-S6	<1	2/1	<0.25	2	8	0.06	**>256**	4	**16**	**>32**	**64**	<0.12/2.38	0.5	**>32**
S7	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**16**	2	**>64**	<0.12/2.38	0.5	**>32**
TC-S7	2	2/1	<0.25	2	8	0.12	**>256**	4	8	**>32**	**64**	<0.12/2.38	0.5	**>32**
S8	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**>16**	2	**64**	<0.12/2.38	0.5	**>32**
TC-S8	2	2/1	<0.25	2	8	0.12	**>256**	2	8	**>32**	**64**	<0.12/2.38	0.5	**>32**
S9	**>32**	**32/16**	**16**	8	8	0.03	256	**32**	0.5	4	4	<0.12/2.38	**>8**	<4
TC-S9	**>32**	**32/16**	**16**	2	8	0.12	<16	**32**	<0.25	**>32**	4	<0.12/2.38	4	<4
S10	**>32**	**32/16**	**16**	8	8	0.03	256	**32**	0.5	4	8	<0.12/2.38	**>8**	<4
TC-S10	**>32**	**32/16**	**16**	2	8	0.25	<16	**>32**	0.5	**>32**	4	<0.12/2.38	4	<4
S12	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	<0.12/2.38	0.5	<4
TC-S12	**>32**	8/4	<0.25	4	8	0.12	**>256**	4	0.5	**>32**	8	<0.12/2.38	1	<4
S13	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	0.25/4.75	0.5	<4
TC-S13	**>32**	8/4	<0.25	4	8	0.12	**>256**	4	0.5	**>32**	8	<0.12/2.38	1	<4
S14	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	<0.12/2.38	1	<4
TC-S14	**>32**	8/4	<0.25	4	8	0.12	**>256**	2	0.5	**>32**	8	<0.12/2.38	1	<4
S15	**>32**	<1/0.5	<0.25	4	8	0.25	**>256**	2	0.5	4	8	<0.12/2.38	1	<4
TC-S15	**>32**	8/4	<0.25	4	8	0.25	**>256**	2	0.5	**>32**	16	0.25/4.75	1	<4
S20	<1	<1/0.5	<0.25	8	8	0.03	64	4	0.5	4	4	<0.12/2.38	1	**>32**
TC-S20	2	2/1	<0.25	4	8	0.12	<16	4	<0.25	**>32**	4	<0.12/2.38	<0.12	**>32**
S24	**>32**	4/2	<0.25	8	8	0.03	**>256**	2	0.5	8	**>64**	<0.12/2.38	1	**>32**
TC-S24	**>32**	8/4	<0.25	4	8	0.12	**>256**	2	0.5	**>32**	**>64**	<0.12/2.38	0.5	**>32**
S27	**>32**	**32/16**	**8**	8	**>32**	<0.015	**>256**	16	0.5	4	**>64**	<0.12/2.38	**8**	**>32**
TC-S27	**>32**	8/4	<0.25	4	**>32**	0.12	**>256**	2	<0.25	**>32**	**32**	<0.12/2.38	0.5	**32**
S28	<1	<1/0.5	<0.25	8	8	<0.015	64	2	<0.25	2	4	<0.12/2.38	1	**>32**
TC-S28	2	2/1	<0.25	8	8	0.12	<16	8	<0.25	**>32**	4	<0.12/2.38	0.5	**>32**
S29	<1	<1/0.5	<0.25	4	8	<0.015	64	2	0.5	4	8	<0.12/2.38	1	**>32**
TC-S29	2	2/1	<0.25	4	8	0.12	<16	2	<0.25	**>32**	<2	<0.12/2.38	0.5	**>32**

[a]*E. coli* transconjugants are indicated by designations beginning with "TC." *Salmonella* isolate designations begin with the letter "S."

[b]MIC ranges of the drugs are as follows: amoxicillin-clavulanic acid (AUG2), 1/0.5 to 32/16 μg/ml (breakpoint, ≥32/16 μg/ml); ampicillin (AMP), 1 to 32 μg/ml (breakpoint, ≥32 μg/ml); azithromycin (AZI), 0.12 to 16 μg/ml (breakpoint, ≥32 μg/ml); cefoxitin (FOX), 0.5 to 32 μg/ml (breakpoint, ≥32 μg/ml); ceftiofur (XNL), 0.12 to 8 μg/ml (breakpoint, ≥8 μg/ml); ceftriaxone (AXO), 0.25 to 64 μg/ml (breakpoint, ≥4 μg/ml); chloramphenicol (CHL), 2 to 32 μg/ml (breakpoint, ≥32 μg/ml); ciprofloxacin (CIP), 0.015 ± 4 μg/ml (breakpoint, ≥4 μg/ml); gentamicin (GEN), 0.25 to 16 μg/ml (breakpoint, ≥16 μg/ml); nalidixic acid (NAL), 0.5 to 32 μg/ml (breakpoint, ≥32 μg/ml); streptomycin (STR), 32 to 64 μg/ml (breakpoint, ≥32 μg/ml); sulfisoxazole (FIS), 16 to 256 μg/ml (breakpoint, ≥512 μg/ml); trimethoprim-sulfamethoxazole (SXT), 0.12/2.38 to 4/76 μg/ml (breakpoint, ≥4/76 μg/ml); and tetracycline (TET), 4 to 32 μg/ml (breakpoint, ≥16 μg/ml). Boldface indicates resistance of the *Salmonella* isolate or transconjugant to the antimicrobial.

Determination of antimicrobial resistance genes. Following the conjugation experiment and AST, 14 AMR-encoding genes were tested using a PCR-based method (Table 3). Only eight of these marker genes, including bla_{CMY-2}, bla_{TEM}, *sul1*, *sul2*, *aadA*, *aadA2*, *tet*(A), and *tet*(B), were detected in plasmids. The bla_{CMY-2} gene was detected in a 100-kb IncI1 plasmid (pS9). The bla_{TEM} gene was found in an IncFII plasmid (pS27). We detected *tet*(A) or *tet*(B) in plasmids that encoded tetracycline resistance. In plasmids carrying streptomycin resistance, *aadA1*, and *aadA2* were found. The *sul1* gene was the

TABLE 3 Primers used for PCR detection of resistance genes

Gene	Forward oligonucleotide sequence (5′ to 3′)	Reverse oligonucleotide sequence (5′ to 3′)	Expected size (bp)	Reference
bla_{CMY-2}	GACAGCCTCTTTCTCCACA	TGGAACGAAGGCTACGTA	1015	76
bla_{PSE-1}	TTTGGTTCCGCGCTATCTG	TACTCCGAGCACCAAATCCG	150	77
bla_{TEM}	GCACGAGTGGGTTACATCGA	GGTCCTCCGATCGTTGTCAG	860	78
aadA	GTGGATGGCGGCCTGAAGCC	AATGCCCAGTCGGCAGCG	528	79
aadA2	CGGTGACCATCGAAATTTCG	CTATAGCGCGGAGCGTCTCGC	250	80
strA	CCTGGTGATAACGGCAATTC	CCAATCGCAGATAGAAGGC	548	79
strB	ATCGTCAAGGGATTGAAACC	GGATCGTAGAACATATTGGC	509	79
sul1	CGGACGCGAGGCCTGTATC	GGGTGCGGACGTAGTCAGC	591	75
sul2	GCGCTCAAGGCAGATGGCATT	GCGTTTGATACCGGCACCCGT	285	78
cmlA	TGGACCGCTATCGGACCG	CGCAAGACACTTGGGCTGC	641	75
tet(A)	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG	210	40
tet(B)	TTGGTTAGGGGCAAGTTTTG	GTAATGGGCCAATAACACCG	659	40
tet(C)	CTTGAGAGCCTTCAACCCAG	ATGGTCGTCATCTACCTGCC	418	40
tet(G)	CAGCTTTCGGATTCTTACGG	GATTGGTGAGGCTCGTTAGC	844	40

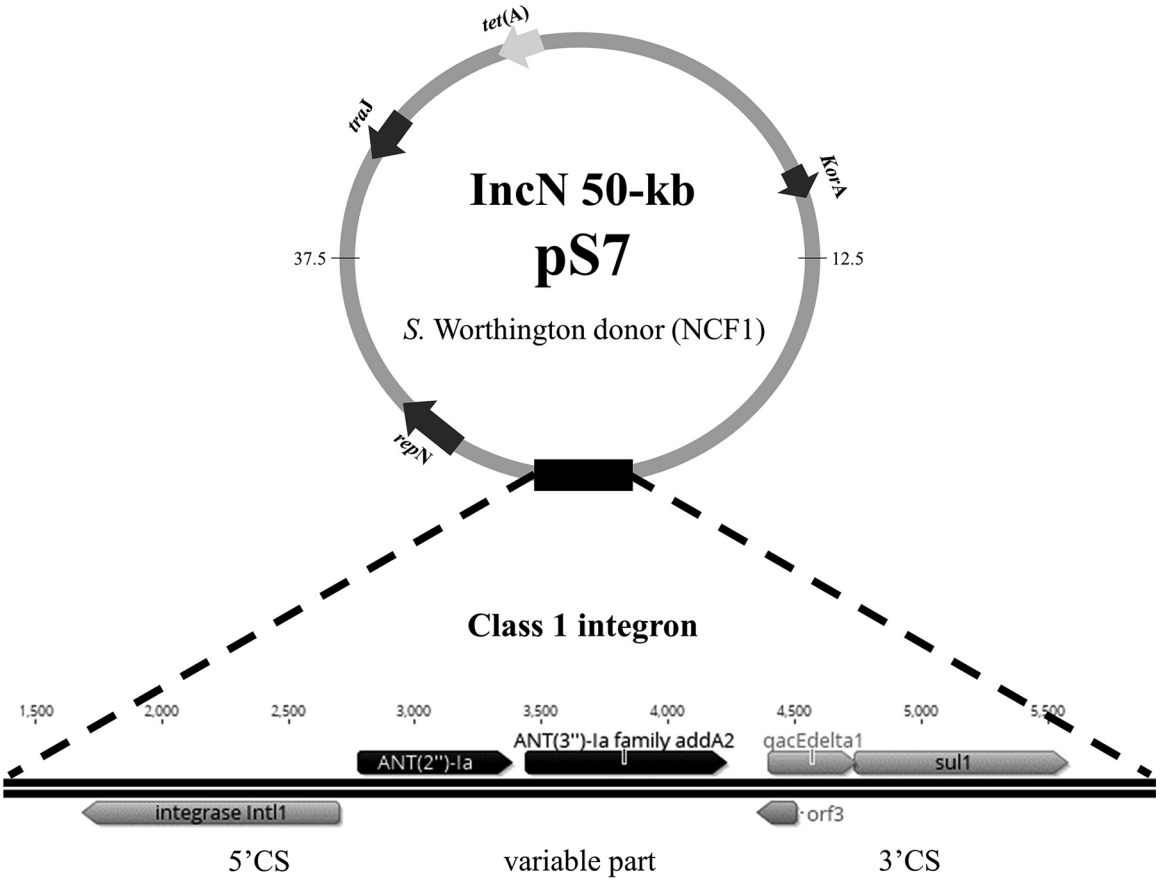

FIG 1 Schematic representation of a class 1 integron in 50-kb IncN plasmid pS7: in the 5′conserved segment, the *intI1* integrase gene; in the variable region, ANT(2″)-Ia, producing the aminoglycoside resistance enzyme, and *aadA2*, an ANT(3″)-Ia family *aadA2* gene producing streptomycin resistance; in the 3′ conserved segment, *qacEΔ1*, a partially deleted gene that encodes quaternary ammonium compound resistance, *sul1*, producing sulfonamide resistance, and *orf3*, of unknown function, on the gene cassette recognized by the integrase. Arrows indicate the direction of the coding sequence.

most prevalent among plasmids which were resistant to the antimicrobial sulfisoxazole. Plasmids pS14 and pS15 did not test positive for any AMR genes which were tested in this study. The resistance genotypes of all 14 plasmids are tabulated in Table 1.

Plasmid sequencing and analysis. The incompatibility (Inc) group and resistance genes of plasmids were confirmed using sequencing (Table 1). Plasmid sequencing was able to identify the replicon families of each individual plasmid. A blastn comparison revealed that 95-kb IncF plasmids from different farms and serotypes (pS9, pS10, pS12, pS13, pS14, pS15, and pS27) (Table 1) were identical to another fully sequenced plasmid, pSTY1-H2662 previously isolated from *S.* Typhimurium from human stool (GenBank accession number CP014980) (33). A class 1 integron was identified in plasmids pS6 to pS8 isolated from *S.* Worthington using *in silico* analysis. This integron was comprised of a 5′ conserved segment (CS), variable part, and 3′ conserved segment (Fig. 1, pS7). The unusual variable part contained an ANT(2″)-Ia-*aadA2* gene cassette, which is responsible for aminoglycoside resistance, while the *sul1* gene was always found in the 3′ CS responsible for sulfonamide resistance. In addition, plasmid sequence analysis revealed the presence of VirB-family type IV secretion systems (T4SS) in all 14 plasmids, together with multiple *tra* genes, including *traC*, *traF*, *traG*, *traI*, *traJ*, *traO*, and *traU*. The evolutionary tree of 14 plasmid sequences was created using Geneious R10 software (Fig. 2). At 70% similarity, the plasmids from the same *Salmonella* donor were clustered together, including pS6, pS7, and pS8 (from *S.* Worthington) and pS28 and pS29 (from *S. enterica* serotype Ohio). The plasmids with distinct sizes, the 100-kb pS9 and 90-kb pS20, were separated from the other group. Plasmid pS24

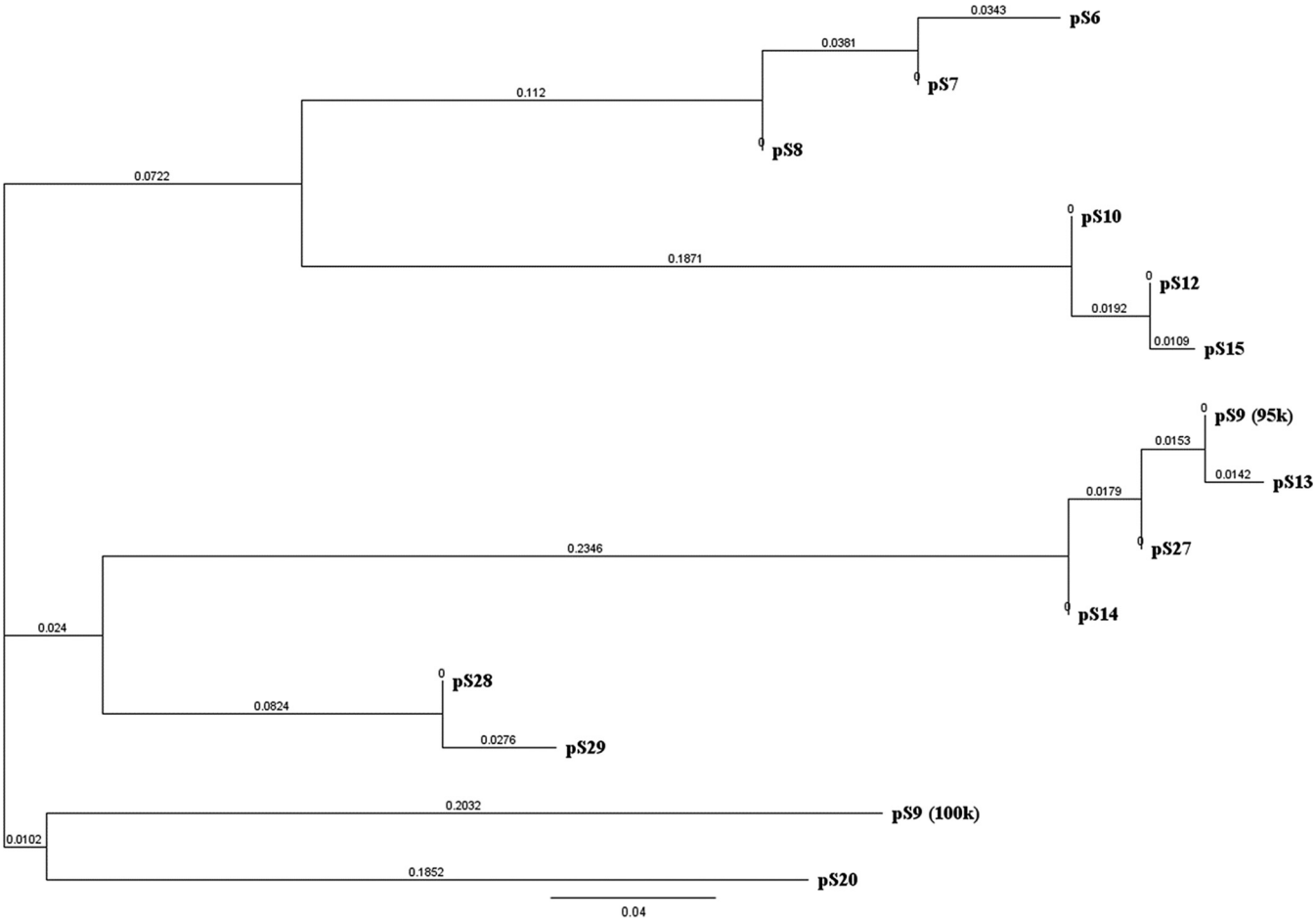

FIG 2 Phylogenetic diversity for sequences of 14 plasmids acquired from environmental *Salmonella* isolates. Evolutionary distances between plasmids were computed using a neighbor-joining algorithm. The distance was obtained from pairwise alignments with 70% similarity and no outgroup. The plasmid label names relate to data in Table 1. Phylogenetic analyses were conducted in Geneious R10.

was not included in the analysis because of the incomplete sequencing output. The plasmid multilocus sequence typing (pMLST) database revealed that three 50-kb IncN plasmids isolated from *S.* Worthington belonged to sequence type 5 (ST5). The IncI1 plasmid (pS9) isolated from *S.* Johannesburg was assigned to ST12 and clonal complex 12 (CC-12); another IncI1 plasmid (pS20) isolated from *S.* Rissen was typed as ST155, but the clonal complex was not defined (Table 1).

DISCUSSION

The aim of the study was to characterize the plasmids identified in different AMR *Salmonella* serotypes isolated from a commercial swine farm environment after land application of manure. We also wanted to determine the role of plasmids in the dissemination of AMR genes to other potential bacterial recipients in the environment. The results potentially addressed the key role played by plasmids in the horizontal gene transfer that leads to the rapid proliferation of AMR genes in the environment. It is important to stress that our study was conducted at commercial swine farms and not at a research station in North Carolina, which is one of the top two leading pork-producing states in the United States. The *Salmonella* serotypes carrying multiple plasmids are common in the *Enterobacteriaceae* family (34). However, we focused on large (defined as being ≥40 kb in size) plasmids which are abundant in *E. coli* and *Salmonella* and comprise important pools of adaptive and transferable genetic information, especially AMR-corresponding genes, in these bacteria (34, 35). The large plasmids, in the range of 40 to 200 kb, have been suggested to be the necessary

markers for extended-spectrum β-lactamases (ESBL), β-lactamase-encoding genes, and plasmid-mediated quinolone resistance (PMQR) (14, 16, 36). In our study, 5 out of 14 plasmids that we detected were 95 kb in size and were isolated from the *S*. Typhimurium var5− serotype ($n = 5$). The plasmid profiles of these five isolates were similar although they were recovered from different farms and at different time points, indicating the persistence of this plasmid in this serotype in the environment after manure deposition. The results correlated with those of a previous study that reported that *Salmonella* plasmids were conserved and primarily serotype specific, including those of *S*. Typhimurium and *S. enterica* serotype Heidelberg, and that they tended to persist for a long period in the environment (34). These plasmids were in contrast to *E. coli* plasmids which were more variable and not specific to particular strains (22, 34). The pS24 plasmid isolated from *S. enterica* 4,12:i:− had a profile similar to that of *S*. Typhimurium plasmids, and the parent strain was also isolated from a different swine farm environment. During the last decade, *S. enterica* 1,4,12:i:−, 1,4,[5],12:i:−, and 4,12:i;− have emerged around the world and have frequently been isolated from human, animal, agricultural production, and environmental sources (37–39). These serotypes are believed to be a mosaic variant of *S*. Typhimurium and are related to plasmid-mediated colistin resistance encoded by the *mcr-1* gene (37, 39, 81). We detected one *S*. Rissen plasmid of approximately 90 kb that carried a tetracycline resistance marker. This is in comparison to our previous report where we identified from a farm environment in North Carolina a 90- to 100-kb plasmid in a tetracycline-resistant *S*. Rissen isolate carrying the *tet*(A) gene (31). This serotype is not common in the U.S. agricultural system and was identified for the first time in North Carolina swine farms in 2009 (42).

Typing of plasmid incompatibility (Inc), the inability of two plasmids of the same family to coexist in the same host cell, classifies plasmids based on their stability during conjugation (43, 82). This classification helps to categorize plasmids into clusters and relies on their phylogenetic relatedness, distribution in the host cells and environment, and their evolutionary origin (43, 44). Currently, 27 Inc groups are identified among the *Enterobacteriaceae* family (43, 45). On the basis of the PCR-based replicon typing (PBRT) method, 18 Inc groups were detected in our study. We used total plasmid DNA from each isolate in conducting PBRT, so the results did not differentiate individual plasmids in multiplasmid isolates. Most of the isolates were positive for more than one replicon family either because the isolates contained multiple plasmids from different incompatibility groups or because a single plasmid carried replication or partitioning genes from more than one incompatibility group. However, we were able to identify the exact replicon families after assessing the plasmid sequencing data (Table 1). We did not differentiate the heterogeneous IncF plasmids into individual groups because of their partitioning of replication genes (34), and the small (<40 kb in size) plasmids were not characterized in this study.

Particular plasmid Inc families, including IncN, IncI1, and IncF, are more frequently associated with the dissemination of AMR genes (14). These three plasmid Inc families have been associated with specific *Salmonella* serotypes and geographic farm areas in our study (34). The IncN family was detected in *S*. Worthington, which was consistently isolated from NCF1, while IncI1 was detected in *S*. Johannesburg isolated from NCF3. Both families are associated with large plasmids related to MDR phenotypes (Table 1). The IncF family was detected in multiple serotypes and farms (NCF3, -5, and -6). These findings are in accordance with those of previous studies that found that IncF and IncI1 are the most prevalent replicon types distributed among the *Enterobacteraceae* (14, 34). The IncI and IncF plasmids generally recovered from *E. coli* and *Salmonella* from human and animal sources are considered the source of several ESBL genes (14, 20, 23).

The IncFI group including FIA, FIB, and FIC, together with the IncFIIA subtype, was the most frequently detected replicon type in this study. All 14 *Salmonella* isolates carried at least one IncF plasmid. Our result supports the view that the IncF (both FI and FII) family was well adapted and commonly distributed in *E. coli* and *Salmonella* (14, 15, 34, 46). Wang et al. (14) reported that IncFIIA was detected only in *S. enterica* serotype

Typhimurium, which correlates with our findings; however, we also detected the FIIA type in the *S. enterica* serotype 4,12:i:−. IncF family plasmids have been reported to contribute to the spread of AMR in *Enterobacteriaceae* and have been associated with specific genes conferring resistance to aminoglycosides, β-lactams, and quinolones (43, 46, 47).

Conjugative plasmids of the IncI1 replicon type were usually associated with multiple resistance compounds, especially extended-spectrum cephalosporinases of both the CTX-M and CMY types (47–49). The IncI1 plasmids carrying TEM-52 have been identified in *E. coli* and *Salmonella* cultured from humans and from chicken and turkey products in the European Union (50–52). The bla_{CMY}-IncI1 plasmids linked to poultry, ground beef, and tomato sources have been identified to be responsible for ceftriaxone-resistant *Salmonella* outbreaks in the United States during 2011 and 2012 (18). Reports indicated that *Salmonella enterica* serotypes Heidelberg, Infantis, Typhimurium, and Newport were associated with IncI plasmids carrying the bla_{CMY} gene. Similar to results of our study, IncI plasmids carrying the bla_{CMY} gene were identified in a ceftriaxone-resistant *S. enterica* serotype Johannesburg isolate from a commercial swine farm environment sampled in our study.

IncN plasmids are the major vehicles for the dissemination of PMQR and ESBL genes, including $bla_{CTX\text{-}M}$ (22, 53, 54). In contrast to results of our study, IncN plasmids were identified in *S*. Worthington transconjugants and exhibited resistance to sulfisoxazole, streptomycin, and tetracycline but not to quinolones and ampicillin. Thus, characterization based on plasmid profiling and the corresponding Inc group using the PBRT technique is an essential tool for plasmid epidemiological surveillance, enhancing discrimination between *Salmonella* serotypes and tracing the spread of AMR genes (14, 16).

Multiple MDR-coding genes were found in plasmids. We detected plasmids carrying *sul1* and *sul2* genes conferring sulfisoxazole resistance, while plasmids with streptomycin resistance carried the *aadA* and *aadA2* genes. Similarly, the *tet*(A) and *tet*(B) genes were found in plasmids in *Salmonella* strains that were resistant to tetracycline. β-Lactamase-encoding (*bla*) genes, including bla_{TEM} and bla_{CMY}, were detected in the plasmids which encoded the resistance to ampicillin and cephalosporin group antimicrobials. Several mechanisms are available for *bla* genes to support HGT between bacteria, thereby ensuring the spread of these markers to new hosts and the environment (14, 55). The heavy use of specific antimicrobials such as tetracycline plays a key role in plasmid dissemination and allows for the selection and enrichment of bacteria with multidrug-resistant plasmids (22, 56, 57).

The class 1 integron with an ANT(2″)-Ia-*aadA2* gene cassette was detected in plasmids pS6 to pS8 retrieved from *S*. Worthington (Fig. 1, pS7). The integron had an unusual organization, with an ANT(2″)-Ia gene cassette which is responsible for resistance against gentamicin (58). The gentamicin resistance was not identified in pS7 but in *Salmonella* isolate S7 (pS7 donor) and pS6 (Tables 1 and 2). After BLAST analysis at NCBI, pS6 to pS8 showed genetic relatedness to a *Klebsiella pneumoniae* MDR IncN plasmid reported from Japan (59). However, the *K. pneumoniae* plasmid harbored different resistance genes than those we detected in the *Salmonella* serotypes from our study. The integrons are able to locate on either a chromosome or a mobile genetic element such as a plasmid (60). Several studies have stated that the integrons harboring *aadA* or a variant of *aadA* genes are common among *Salmonella* species (10, 61–63). The variable parts of integrons might be composed of variants of *aad*, *dfr*, or *bla* genes that contribute to aminoglycoside, sulfonamide, and cephalosporin resistance, respectively (10, 61). *S. enterica* serotype Worthington detected in our study is commonly found in poultry, poultry products, and the environment in several parts of the world and harbors integrons either on the chromosome or plasmids (62, 64–66). The presence of genetic elements such as integrons, transposons, and plasmids has consequently been associated with multidrug resistance phenotypes among *Salmonella* isolates (10). Our study reports an emerging multidrug-resistant clone isolated from *Salmonella*

serotypes in a commercial swine farm environment carrying a large conjugative plasmid with an ANT(2″)-Ia-*aadA2* gene cassette located on an integron.

Though the *Salmonella* plasmids were transferred to an *E. coli* JM109 recipient under laboratory conditions, the presence of VirB-family type IV secretion systems (T4SS) and *tra* genes in our study confirms that HGT by conjugation is likely to occur in the environment. The T4SS in Gram-negative bacteria functionally encompass the conjugation system and the effector translocators for interbacterial transfer of AMR genes, virulence determinants, and genes encoding other traits beneficial to the host (67). IncN plasmids (pS6 to pS8) and IncI1 plasmids (pS9 [100-kb] and pS20) employed TraJ, which the has ability to conjugate, and the conjugation process could be stimulated approximately 100-fold, demonstrating functional conservation of a significant regulatory feature of F-like conjugation modules (68).

The phylogenetic tree of 14 plasmids (Fig. 2) at 70% similarity suggested that the plasmids analyzed in our study were clustered based on the *Salmonella* donor serotypes, such as the *S*. Worthington cluster (pS6 to pS8) and *S*. Ohio cluster (pS28 and pS29). Within three Inc groups (IncI1, IncN, and IncF), the phylogenetic analysis also suggested the existence of an Inc group that is serotype specific (34). Based on the pMLST database, all IncN (pS6 to pS8) plasmids which were specific to *S. enterica* serotype Worthington belonged to the same ST5. These results were in accordance with the BLAST output for individual plasmids and the *Salmonella* clustering done by pulsed-field gel electrophoresis (PFGE) in our previous study (13).

Our study demonstrated that identical plasmids were recovered from different *Salmonella* serotypes isolated either from the same or different farm environments. Our findings provide evidence of a single, large 95-kb IncF plasmid being distributed across the swine production systems in North Carolina among different serotypes of *Salmonella*. In addition, we found that AMR plasmids were able to persist in the swine farm environment after manure application for a minimum period of 21 days (final sampling time point). The AMR determinants on these plasmids were transferable among *Salmonella* serotypes, which underlined the fact that manure deposition enriches the environmental resistome. We recommend conducting longitudinal studies on commercial food animal farms to determine the role of manure deposition on the environmental dissemination of AMR genes.

MATERIALS AND METHODS

***Salmonella* serotype selection.** A total of 168 AMR *Salmonella* isolates from commercial swine farm environments in North Carolina during 2013 to 2015 were tested for their plasmid components. The details of farm distribution, waste management systems, sample collection, and *Salmonella* isolation were described in a previous study (13). Briefly, manure samples from a lagoon and soil samples before and after manure spray application were collected on the first day (day 0) of the farm visit. The subsequent soil samples were collected on day 7, day 14, and day 21 from the same plots as on day 0. The serotyping, antimicrobial susceptibility testing (AST), and pulsed-field gel electrophoresis (PFGE) were performed for phenotypic and genotypic characterization of the *Salmonella* strains. The *Salmonella* isolates selected for plasmid characterization were chosen based on their temporal and spatial relationships, AMR profiles, AMR determinants, and PFGE fingerprint profiles. Based on the above criteria, a total of 14 isolates were finally selected for plasmid analysis and sequencing (Table 1). All isolates were maintained at −80°C in brucella broth (Difco, Becton-Dickinson, USA) until further characterization.

Conjugation experiments. Conjugation experiments were conducted to evaluate intra- and interserovar transmission of AMR genes among AMR *Salmonella* serotypes. Fourteen AMR *Salmonella* isolates were selected to serve as donor strains, and the nalidixic acid-resistant (NAL^r) *Escherichia coli* JM109 strain was used as a recipient strain. A heat shock assay modified from Zeng et al. (69) was utilized for performing conjugation experiments. In brief, a loopful of overnight culture of the donor strain was gently mixed in Luria-Bertani (LB) broth (Difco, Becton-Dickinson, USA) with *E. coli* JM109. The donor and recipient DNA mixtures were kept on ice for 20 to 30 min, given heat shock in a water bath at 42°C for 30 to 60 s, and moved back on ice for 2 min. We added 250 to 1,000 μl of LB broth and incubated the culture mix at 37°C in a shaking incubator for 45 to 60 min. The culture mixtures were transferred to selective LB plates (Criterion; Hardy Diagnostics, USA) containing nalidixic acid (50 μg/ml) and one of the antimicrobials, depending on the resistance profile of the donor strain, and incubated at 37°C overnight. Transconjugants were confirmed on nontyphoidal *Salmonella* chromogenic plates (CHROMagar, Paris, France) and xylose lactose tergitol (XLT4) agar plates (Criterion; Hardy Diagnostics, USA). The antimicrobials and the concentrations used are as follows: ampicillin, 100 μg/ml; nalidixic acid, 50 μg/ml; and tetracycline, 20 μg/ml.

Antimicrobial susceptibility testing. The transconjugant AMR and MIC profiles were determined by the broth microdilution method using a Gram-negative Sensititre (CMV3AGNF) plate (Trek Diagnostic Systems, OH). The panel of 14 antimicrobials tested include amoxicillin-clavulanic acid (AUG2; 1/0.5 to 32/16 μg/ml), ampicillin (AMP; 1 to 32 μg/ml), azithromycin (AZI; 0.12 to 16 μg/ml), cefoxitin (FOX; 0.5 to 32 μg/ml), ceftiofur (XNL; 0.12 to 8 μg/ml), ceftriaxone (AXO; 0.25 to 64 μg/ml), chloramphenicol (CHL; 2 to 32 μg/ml), ciprofloxacin (CIP; 0.015 to 4 μg/ml), gentamicin (GEN; 0.25 to 16 μg/ml), nalidixic acid (NAL; 0.5 to 32 μg/ml), streptomycin (STR; 2 to 64 μg/ml), sulfisoxazole (FIS; 16 to 256 μg/ml), trimethoprim-sulfamethoxazole (SXT; 0.12/2.38 to 4/76 μg/ml), and tetracycline (TET; 4 to 32 μg/ml). The MICs were determined, and breakpoints were interpreted based on the Clinical and Laboratory Standards Institute standards (CLSI) for broth microdilution (70, 71) and the National Antimicrobial Resistance Monitoring System (NARMS) (72). *E. coli* ATCC 25922 was used as a quality control strain. The transconjugants with MICs in the intermediate level were categorized as susceptible to avoid overestimation of resistance. The transconjugants with resistance to three or more classes of antimicrobials were classified as multidrug resistant (MDR).

Plasmid isolation. Plasmid DNA was isolated from the confirmed transconjugant (NAL^r *E. coli* JM109) cultures by the modified alkali lysis method described by Sambrook et al. (73), which is suitable for the isolation of both large and small plasmids. The purified DNA concentrations of the plasmid extracts were calculated by measuring the absorbance at 260 and 280 nm using a NanoDrop ND-2000 Spectrophotometer (NanoDrop; Wilmington, DE) and Qubit, version 3.0, fluorometer (Invitrogen, Carlsbad, CA) to ensure that there was adequate plasmid DNA for sequencing. The plasmid DNA was stored frozen at −20°C until required.

PCR amplification of resistance genes. The presence of resistance genes on plasmids of specific AMR *Salmonella* phenotypes was detected using PCR (31, 74). Overall, genes encoding resistance to ampicillin and cephalosporin (bla_{PSE-1}, bla_{TEM}, and bla_{CMY-2}), chloramphenicol (*cmlA*), streptomycin (*aadA1*, *aadA2*, *strA*, and *strB*), sulfisoxazole (*sul1* and *sul2*), and tetracycline [*tet*(A), *tet*(B), *tet*(C), and *tet*(G)] were tested. Template plasmid DNAs were extracted by the modified alkali lysis method mentioned above. The primers, amplicon sizes, and references used to detect the presence of the selected AMR genes are listed in Table 3. The PCR conditions for all resistance genes, except the *cmlA* and *sul1* genes, included an initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 54°C, extension for 1 min at 72°C, and a final extension at 72°C for 7 min. For the *cmlA* and *sul1* genes, the PCR conditions used have been described previously (75). Briefly, an initial denaturation at 94°C for 5 min was followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 57°C, extension for 1 min at 72°C, and a final extension at 72°C for 5 min. *Salmonella enterica* isolates carrying resistance genes and characterized in earlier studies were used as positive controls (31).

Plasmid PCR-based replicon typing (PBRT). Single and multiplex PCRs were run to identify different incompatibility (Inc) groups, including FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA. The primers and PCR running conditions have been described in a previous study (45). The purified plasmid DNA from the modified alkali lysis method was used as the template DNA. PCR running conditions used for the five multiplex PCRs and three single PCRs included an initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 60°C, and elongation for 1 min at 72°C, with a final extension of 5 min at 72°C. The single PCRs for F_{rep} were performed under the same amplification conditions but with an annealing temperature of 52°C. The PCR products were electrophoresed on a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer and UV visualized by staining with ethidium bromide.

Plasmid sequencing, assembly, and annotation. Isolated plasmid DNA libraries were prepared for sequencing using a Nextera XT kit (Illumina, San Diego, CA). Multiplexed sequencing of these libraries was done with a single run on an Illumina MiSeq using 2-by-250- or 2-by-300-bp paired-end reads (MiSeq reagent kit, version 3). Following demultiplexing, sequences were analyzed using CLC Genomic Workbench 10 (Qiagen, Valencia, CA). For analyzing plasmid content, *de novo* assembly of unused reads into new contigs was applied. The initially assembled contigs were analyzed using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST). In addition, individual sequence reads were mapped back to the assembled plasmids to confirm that there were continuous overlapping reads over the entire length of the assembled plasmid. Following completion of plasmid assembly, the plasmid sequences were run through a BLAST search individually and compared to GenBank sequences. The open reading frame (ORF) of each gene in plasmid contigs was identified, and the particular genes of interest were annotated using Geneious R10 software (BioMatters, New Zealand). Manual trimming and editing of terminally redundant contig ends generated circular plasmid genomes. The complete plasmid sequences were visualized using plasmid mapping in the CLC Workbench and deposited in the GenBank under prospective accession numbers.

Comparative genotypic analysis. To further characterize the plasmids and compare their profiles, we mapped the PCR primers described by Carattoli et al. (45) to the assembled plasmid sequences with a BLAST search configured for short reads. Based on the annotations and BLAST output, the plasmids were assessed for the presence of known AMR genes, plasmid transfer (*tra*) genes, and mobile genetic elements, including class I integrons and transposons. The assembled plasmid sequences submitted to a BLAST search were compared to previously sequenced plasmids in GenBank. We identified 14 plasmid sequences and analyzed them for variation using the Geneious R10 software (BioMatters, New Zealand) global alignment with 70% similarity to construct neighbor-joining trees using the Tamura-Nei genetic distance model. In addition, all 14 plasmid sequences were typed by pMLST as previously described (41) and assigned to STs according to the plasmid MLST database (https://pubmlst.org/plasmid/) for ST prevalence analysis.

Accession number(s). The sequencing output of the 14 *Salmonella* plasmids was submitted to the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA293224. Individual plasmid sequence reads have been deposited in the Sequence Read Archive (SRA) as BioSample numbers SAMN07345795 to SAMN07345807.

ACKNOWLEDGMENTS

We gratefully acknowledge the swine producers in the state of North Carolina for allowing access to their farms for collecting the manure and environmental samples. This study would not have been possible without their cooperation and support. We thank Joy Horovitz, who assisted in the plasmid sequencing.

This project was funded by the National Pork Board grant (project number 556678) and the College of Veterinary Medicine, North Carolina State University.

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