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Transmission of extended-spectrum cephalosporin-resistant *Salmonella* harboring a *bla*$_{CMY-2}$-carrying IncA/C$_2$ plasmid chromosomally integrated by IS*Ecp1* or IS*26* in layer breeding chains in Japan

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ABSTRACT. Dissemination of extended-spectrum cephalosporin (ESC)-resistant *Salmonella* is a public health concern in the egg production industry. ESC-resistant *Salmonella* often acquires the *bla* gene via insertion sequences (ISs). Therefore, this study aimed to assess antimicrobial resistance in *Salmonella* from Japanese layer breeding chains and egg processing chains, and determine the genetic profiles of IS-like elements in ESC-resistant *Salmonella*. Antimicrobial susceptibility testing was performed on 224 isolates from 49 facilities involving layer breeder farms, hatcheries, pullet-rearing farms, and layer farms in breeding chains along with egg processing chains. ESC-resistant *Salmonella* strains were whole-genome sequenced. Among them, 40 (17.9%) were resistant to at least streptomycin, tetracycline, ampicillin, chloramphenicol, cefpodoxime, nalidixic acid, ciprofloxacin, and/or kanamycin despite lacking resistance to azithromycin and meropenem. Moreover, 15 were ESC-resistant *Salmonella* harboring *bla*$_{CMY-2}$ (*Salmonella enterica* serovar Ohio, n=12; *S.* Braenderup, n=1; untypeable with O7:b:-, n=1) and *bla*$_{CTX-M-14}$ (*S.* Cerro, n=1). IncA/C$_2$ plasmids containing IS*Ecp1*, IS*26*, and multiple antimicrobial resistance genes (including *bla*$_{CMY-2}$) were identified in *S.* Ohio isolates from pullet-rearing and layer farms belonging to the same company. Chromosomal integration of partial or whole IncA/C$_2$ plasmids was seen with two *S.* Ohio isolates via IS*Ecp1* or IS*26*, respectively. Antimicrobial resistance genes such as *bla*$_{CMY-2}$ might be transmitted among the upper and the lower levels of layer breeding chains via the replicon type IncA/C$_2$ plasmids containing IS*Ecp1* and IS*26*.

KEY WORDS: *bla*$_{CMY-2}$, chromosomally-integrated IncA/C$_2$ plasmid, IS*26*, layer breeding chain, *Salmonella*

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Investigations into antimicrobial-resistant *Salmonella* in the egg production industry are essential because dissemination of this bacterium in this industry is a public health concern. Extended-spectrum cephalosporin (ESC) treatment is required for severe infections with antimicrobial-resistant *Salmonella* in humans [10]. No data, however, are currently available about the incidence of chicken egg-borne salmonellosis in Japan despite the consumption of eggs and egg products being one of the major causes of human salmonellosis worldwide [38]. Therefore, better knowledge and understanding about the prevalence of antimicrobial-resistant *Salmonella* in contaminated eggs and egg products are important. Vertical transmission of *Salmonella* occurs in layer chickens in breeding chains [14, 15, 30]. The chains have upper levels (breeder farms, hatcheries, and pullet-rearing farms) and a lower level (layer farms) [7, 35]. To the best of our knowledge, only two studies have been conducted on antimicrobial-resistant *Salmonella* in layer breeding chains and shell and liquid egg processing chains (LB-EP chains) in Japan. These studies reported that the environments within the layer farms and the shell egg grading and packing centers (EGPs) contained *Salmonella* with resistance to antimicrobials such as ampicillin (ABPC), streptomycin (SM), and cephalothin [11, 32]. Moreover, the EGPs are key factors relating to *Salmonella* transmission in egg production. Therefore, investigating the prevalence of antimicrobial-resistant *Salmonella*, especially ESC-resistant *Salmonella*, in the LB-EP chains, including the upper levels of the layer breeding chains, is important.

ESC-resistant *Salmonella* is generated when *bla* genes encoding AmpC β-lactamase and extended-spectrum β-lactamase (ESBL) are acquired by *Salmonella* [8, 27–29]. *bla* genes translocate between plasmids and chromosomes containing insertion sequences (ISs) among Enterobacteriaceae, including *Salmonella* [8, 23, 27, 29]. In fact, $bla_{\text{CTX-M-14}}$ was translocated by IS*Ecp1* from a plasmid into another plasmid in *Salmonella enterica* subsp. *enterica* serovar Senftenberg (*S.* Senftenberg), whereas $bla_{\text{CTX-M-15}}$ was translocated by IS*Ecp1* from a plasmid into *S.* Haardt chromosomes [29]. Obtaining further knowledge about IS*26* is also important because IS*26* may generate multiple antimicrobial-resistant *Salmonella* via gene integration [9, 23, 27]. A field study indicated that nine antimicrobial resistance genes, including *tet(A)*, *sul2*, *floR*, and $bla_{\text{CMY-2}}$, in an IncA/C plasmid were simultaneously integrated into the *S.* Typhimurium chromosome by IS*26* [27]. A more recent laboratory study showed that IS*26* can translocate $bla_{\text{TEM-1}}$, *sul2*, and *strAB* from the *S.* Typhimurium chromosome into its plasmid [23]. These studies suggest that IS*26* may be associated with the generation of multiple antimicrobial-resistant *Salmonella*. Therefore, understanding the emergence and dissemination of ESC-resistant *Salmonella* requires the investigation of ISs such as IS*Ecp1* and IS*26* on *bla* gene-carrying plasmids and chromosomes. The aims of this study were as follows: (i) assessing *Salmonella*-specific antimicrobial resistance patterns in LB-EP chains, especially ESC-resistant *Salmonella*; (ii) determining plasmid and chromosome encoded features in *Salmonella* including ISs and IS-related *bla* genes using whole-genome sequencing (WGS); and (iii) confirming whether IS*26* generates a multiple antimicrobial resistance phenotype in *Salmonella*.

MATERIALS AND METHODS

Samples from eggshells and egg production environments

A total of 224 samples were collected from one breeder farm (n=4), two hatcheries (n=2), five pullet-rearing farms (n=20), 39 layer farms (n=194), and two EGPs (n=4) in Japanese LB-EP chains between 2009 and 2016, excluding 2014 (Table 1). From them, 51, 43, 41, 43, 40, 3, and 3 were gathered in 2009, 2010, 2011, 2012, 2013, 2015, and 2016, respectively. These 49 facilities are located in seven prefectures (A, B, C, D, E, F, and G), with six of the seven located in western Japan (prefectures A–F), and the remaining one in eastern Japan (prefecture G).

The 224 samples were pooled eggshell samples (n=89) and environmental egg production samples (n=135; swab, n=77; dust, n=50; feces, n=5; unidentified, n=3) from facilities in the LB-EP chains. The types of facility where these samples were collected are shown in Table 1. Each pooled eggshell sample consisted of 100–150 eggshell remnants from liquid egg production on each farm. Environmental sampling of the egg production facilities was conducted as follows. Dust clouds were collected from the ventilators attached to the pullet-rearing farms and layer farms, and feces were collected from a layer breeder farm or a hatchery. Swabs were also obtained from the swabbing floors of all the facilities other than the EGPs, and from the swabbing floors and egg containers in the EGPs. The swabs were gathered using swabbing sheets (Kanto Chemical Co., Inc., Tokyo, Japan) and cotton gauze swabs (Eiken Chemical Co., Tokyo, Japan).

Table 1. Sample information for the *Salmonella* isolates obtained from upper[a] and lower[b] levels in layer breeding chains and egg shell grading and packing centers (EGPs)

Facility type	No. of facilities	No. of isolates	No. of samples				
			Pooled eggshell	Swab	Dust	Feces	Unidentified
Layer breeder farm	1	4				4	
Hatchery	2	2		1		1	
Pullet-rearing farm	5	20		14	5		1
Layer farm	39	194	89	58	45		2
EGP	2	4		4			
Total	49	224	89	77	50	5	3

[a] Upper levels consist of layer breeder farms, hatcheries, and pullet-rearing farms. [b] Lower levels consist of layer farms.

Salmonella were isolated from the samples in accordance with a previous description with minor revision [17, 28]. Each sample, except for the pooled eggshell samples, was mixed with 225 ml of buffered peptone water (BPW, Oxoid Ltd., Hampshire, UK), and then tapped by hand for 1 min. The mixed BPW was cultured (37°C, 20 ± 2 hr). The pooled eggshell samples were cultured in 1,500–2,000 ml of BPW (37°C, 20 ± 2 hr). An aliquot (0.1 ml) of the BPW culture was added to Rappaport-Vassiliadis enrichment broth (Oxoid Ltd.) and cultured (42°C, 20 ± 2 hr). The cultured broth was streaked onto mannitol, lysine, crystal violet and brilliant green agar (Oxoid Ltd.) and Rimler-Shotts-Maeda agar (Kanto Chemical Co.) at 37°C for 18–48 hr for bacterial isolation. Four putative *Salmonella* colonies were picked and then identified as *Salmonella* by biochemical tests following the previous reports [16, 28, 29]. Serological tests were conducted in accordance with our previous description [16, 28, 29].

Antimicrobial susceptibility and phenotype testing

Antimicrobial susceptibility tests were performed using the disk diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [4]. The susceptibility test results were evaluated in accordance with the CLSI criteria for resistance breakpoints [5]. The following 10 antimicrobials were used: ABPC (10 µg), cefpodoxime (CPDX, 10 µg), chloramphenicol (CP, 30 µg), ciprofloxacin (CPFX, 5 µg), nalidixic acid (NA, 30 µg), kanamycin (KM, 30 µg), SM (10 µg), azithromycin (AZM, 15 µg), tetracycline (TC, 30 µg), and meropenem (MEPM, 10 µg). The antimicrobial disks came from Becton, Dickinson, and Co. (Franklin Lakes, NJ, USA). Regarding the isolates showing resistance to ABPC, ESBL-producing confirmatory tests were performed using ceftazidime (CAZ) (30 µg)-clavulanic acid (CVA) (Becton, Dickinson, and Co.), CTX (30 µg)-CVA (Becton, Dickinson, and Co.), and CPDX (30 µg)-CVA (Eiken Chemical Co.) disks, as per our previous reports [28, 29]. We also performed boronic acid tests using CPDX and cefoxitin disks as per our previous reports [28, 29]. *Escherichia coli* strain ATCC 25922 was used as the quality control strain in all the assays in accordance with CLSI guidelines [5]. *Klebsiella pneumoniae* ATCC 700603 and *E. coli* ATCC 35218 were also used as quality control strains.

The minimum inhibitory concentrations (MICs) of ABPC, ampicillin-sulbactam, CAZ, CPDX, CTX and ceftriaxone antimicrobials were evaluated against the four strains (three *S.* Ohio strains: SEOhiM1593, 1960, and 2008, and one *S.* Cerro strain: SECerM2017) using the Etest (bioMérieux, Marcy-l'Étoile, France), which was performed in accordance with the manufacturer's instructions.

Detection and sequencing analysis of β-lactamase genes

Polymerase chain reactions (PCRs) were conducted as previously described to detect β-lactamase genes (bla_{CMY}, bla_{CTX-M}, bla_{SHV}, and bla_{TEM}) in the isolates that were resistant to ABPC [22, 28, 29]. The presence of the *bla* gene was determined by DNA sequencing as described in a previous study with minor revisions using the primers shown in Supplementary Table 1 [22]. PCR products were purified using the illustra ExoProStar enzymatic PCR and Sequencing Clean-Up kit (Global Life Sciences Solutions USA LLC., Marlborough, MA, USA). DNA sequencing was performed using the BigDye Terminator Cycle Sequencing Reaction v. 3.1 kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the products were analyzed on the 3500 Genetic Analyzer (Thermo Fisher Scientific Inc.). The DNA sequences were analyzed by comparison with the reference sequences registered in the National Center for Biotechnology Information (NCBI) database [19].

Epidemiological information on bla gene-carrying Salmonella strains

To elucidate the relationship(s) involving the dissemination of ESC-resistant, *bla* gene-carrying *Salmonella* between the lower-level facilities and upper-level facilities in the layer breeding chains, we collected detailed information about the strains, such as the facility type and the isolation date (Supplementary Table 2).

WGS on Salmonella harboring bla_{CMY-2} or $bla_{CTX-M-14}$ and Salmonella genome analysis

WGS was performed to obtain the complete chromosomal and plasmid DNA sequences of three *S.* Ohio strains harboring bla_{CMY-2} (SEOhiM1593, 1960, and 2008) and one *S.* Cerro strain harboring $bla_{CTX-M-14}$ (SECerM2017) in accordance with the methods used in our previous study with minor revisions [18]. To confirm the genetic features of *Salmonella* harboring bla_{CMY-2} or $bla_{CTX-M-14}$, we selected four strains for WGS analysis. *Salmonella* strains harboring bla_{CMY-2} were selected from the bla_{CMY-2}-carrying strains derived from both layer farms and pullet-rearing farms belonging to the same layer breeding chains. Of the *Salmonella* strains harboring bla_{CMY-2}, two *S.* Ohio strains were selected at random from the 11 bla_{CMY-2}-carrying strains derived from pullet-rearing farms and one *S.* Ohio was selected from a layer farm. We also selected the *S.* Cerro strain (SECerM2017) for analysis because it was the only $bla_{CTX-M-14}$-carrying strain. Total DNA was extracted from each strain using the MagAttract HMW DNA Kit (Qiagen, Venlo, Netherlands). The extracted DNA was barcoded (Native Barcoding Expansion 1–12; Oxford Nanopore, Oxford, UK), and made into a nanopore-sequencing library with Ligation Sequencing 1D Kit (Oxford Nanopore). The library was sequenced on R9.4.1 flowcells using the MinION portable DNA sequencer (Oxford Nanopore). We also performed Illumina sequencing on the extracted genomic DNA preparations remaining from the nanopore-sequencing. Libraries were prepared using the Nextra DNA Flex Library Prep Kit (Illumina, Inc., San Diego, CA, USA). Cycles of 300 or 500 dual-index paired-end sequencing were performed using Illumina MiSeq (Illumina, Inc.). Complete chromosomes and plasmids from the three *S.* Ohio strains and the single *S.* Cerro strain were generated from the Nanopore and Illumina Sequencing data using Unicycler v.0.4.4 or Flye v.2.4.2 for *de novo* assembling and Pilon v.1.23 for polishing [13, 36, 37]. The complete chromosomal sequences and plasmid sequences were analyzed using nucleotide BLAST (BLASTN) homology searches in PlasmidFinder and ResFinder to infer the plasmid replicon types and the antimicrobial resistance genes as per our previous study [1, 12, 28]. The DDBJ Fast Annotation and Submission Tool (DFAST) annotation pipeline in the DNA Data Bank of Japan (DDBJ) was used for genome annotation [20].

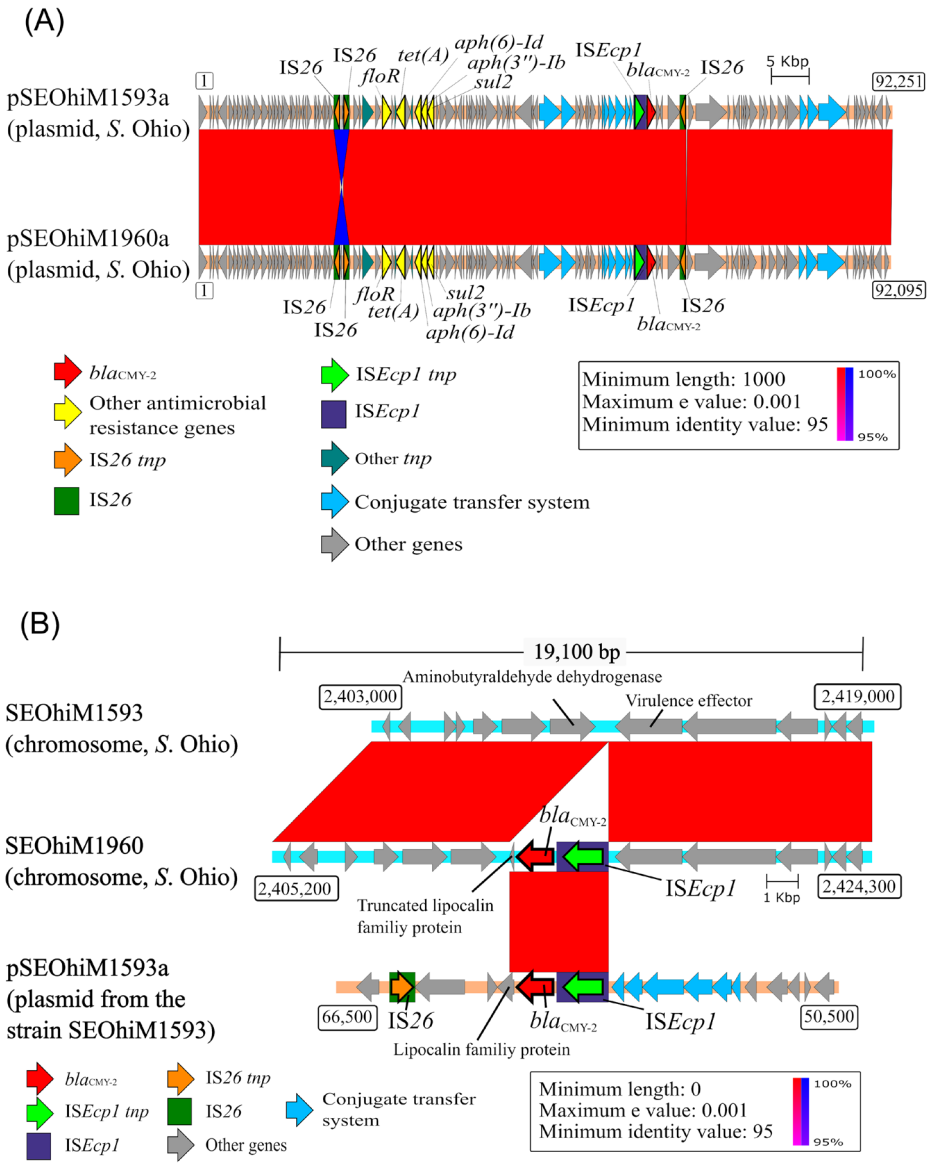

Fig. 1. **(A)** Structural comparison of pSEOhiM1593a and pSEOhiM1960a plasmids. pSEOhiM1960a is derived from a layer farm and shares high similarity with pSEOhiM1593a derived from a pullet-rearing farm in the same breeding chain of the same company in different years. These plasmids harbor six antimicrobial resistance genes, one IS*Ecp1*, and three IS*26* (Table 3). Numbers in rectangular boxes are nucleotide positions. **(B)** Structural comparison of the bla_{CMY-2}-carrying-chromosome in strain SEOhiM1960. A chromosomal region carrying bla_{CMY-2} along with IS*Ecp1* is completely identical to a part of pSEOhiM1593a. Numbers in rectangular boxes are nucleotide positions. **(C)** Structural comparison of the *Salmonella enterica* subsp. *enterica* serovar Ohio chromosome from strain SEOhiM2008, which carries multiple antimicrobial-resistant genes (Table 3). The pSEOhiM1593a plasmid is drawn to show the rearrangement at the start position (from the *rep* gene-encoding plasmid's replication protein). The black bullet point indicates the position of the *rep* gene. Numbers in rectangular boxes are nucleotide positions. A region similar to intact pSEOhiM1593a was detected on the SEOhiM2008 strain's chromosome, although the region sandwiched between the two IS*26* is inverted. This region is flanked by two IS*26* elements, and a couple of target duplication sites of 8 bp in length were also detected, suggesting that IS*26* integrated this region into the chromosome. **(D)** Horizontal transmission of positively related plasmids (pSEOhiM1593a and its highly related plasmids) between *S.* Ohio strains, and integration of these plasmids into the chromosomes of these strains via insertion sequences IS*Ecp1* or IS*26*. The plasmids were transmitted horizontally from the upper levels (pullet-rearing farm) to the lower level (layer farm) in a layer breeding egg production chain within the same company. A part of the whole plasmid or its entirety was integrated into the *S.* Ohio chromosomes by IS*Ecp1* or IS*26*, respectively.

Comparative genome sequence analysis

The nucleotide sequences from two bla_{CMY-2}-carrying plasmids (pSEOhiM1593a and 1960a) and those from the chromosomes of the three *S.* Ohio strains (SEOhiM1593, 1960, and 2008) were used for structural genome comparisons (Fig. 1). The pSEOhiM1593a plasmid (recovered from strain SEOhiM1593) was compared with the pSEOhiM1960a plasmid (recovered from strain SEOhiM1960) (Fig. 1A). The bla_{CMY-2}-containing SEOhiM1960 chromosome and the bla_{CMY-2}-lacking SEOhiM1593

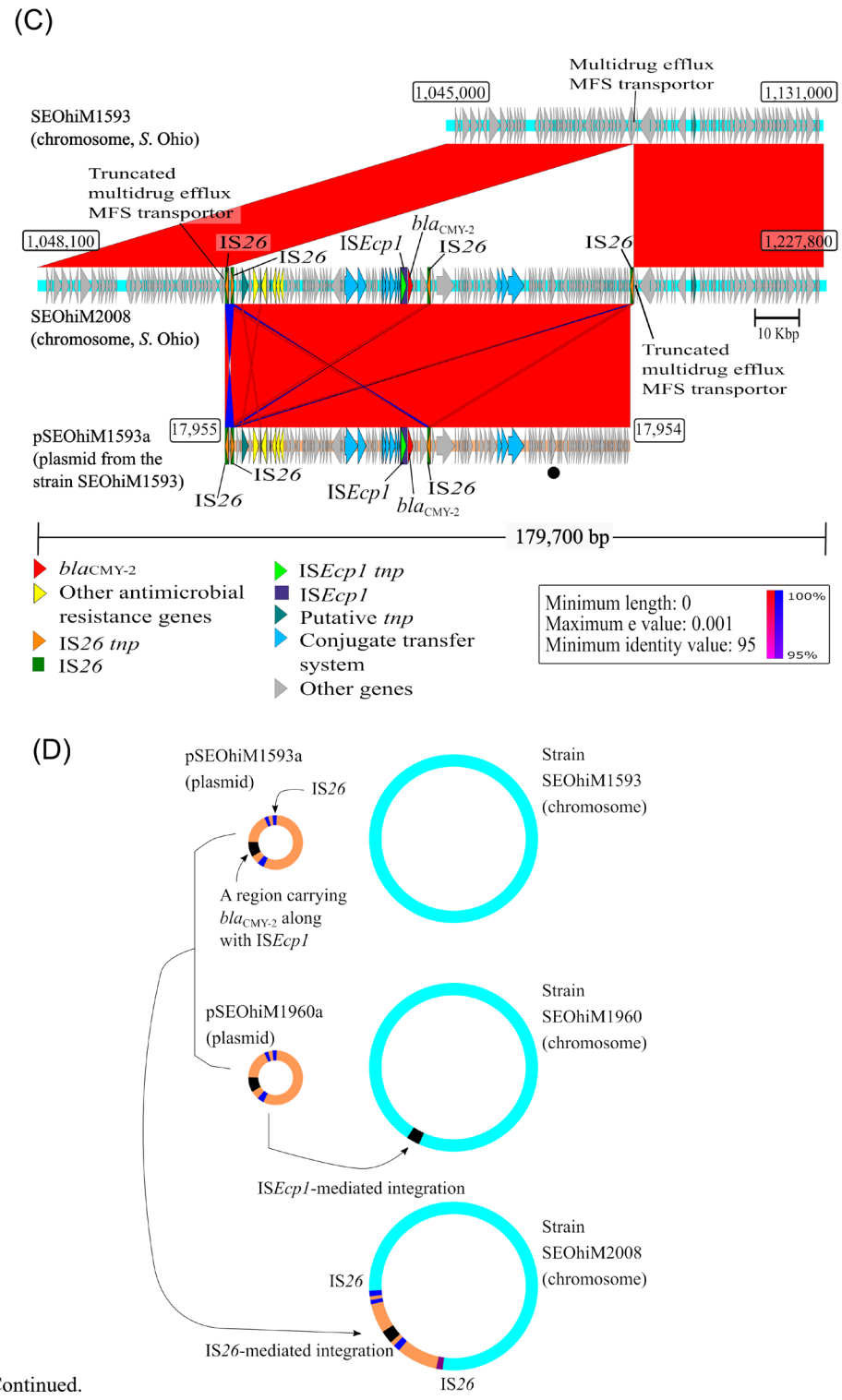

Fig. 1. Continued.

chromosome were used for comparison; they were also used for another comparison with the pSEOhiM1593a plasmid (Fig. 1B). The SEOhiM2008 chromosome was compared in the same manner as that of the SEOhiM1960 chromosome (Fig. 1C). Additionally, a genome structure comparison of the $bla_{\text{CTX-M-14}}$-harboring *S.* Cerro (SECerM2017) chromosome was performed. The chromosomal nucleotide sequence from SECerM2017 was compared with that from SEOhiM1593 (Fig. 2). We also compared the SECerM2017 chromosome with the complete sequence of the p14E509-CTXM plasmid (GenBank accession no. MG764547.1) [39]. We did this because the BLASTN analysis revealed that this plasmid shares the highest nucleotide sequence similarity score (coverage 100%) with a $bla_{\text{CTX-M-14}}$-containing region in SECerM2017. In common with the other genome comparisons, the sequence of the p14E509-CTXM reference plasmid was annotated using DFAST. Comparative genome sequence analysis was performed using

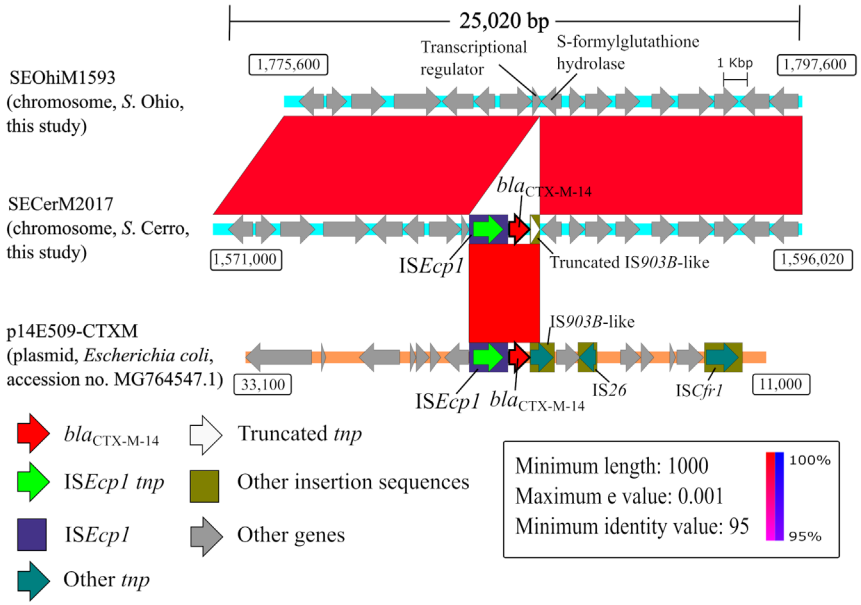

Fig. 2. Structural comparison of the $bla_{CTX\text{-}M\text{-}14}$-carrying chromosome from *Salmonella enterica* subsp. *enterica* serovar Cerro (SECerM2017) versus the analogous chromosomal region from *S.* Ohio (SEOhiM1593). The region carrying $bla_{CTX\text{-}M\text{-}14}$ along with IS*Ecp1* in the chromosome is identical to a $bla_{CTX\text{-}M\text{-}15}$-carrying part of plasmid p14E509-CTXM from a clinical *Escherichia coli* isolate from China (GenBank accession no. MG764547.1). Numbers in rectangular boxes are nucleotide positions.

BLASTN and the Artemis Comparison Tool [2], and the results were visualized using Easyfig [33]. ISs were confirmed in the analyzed plasmids using ISfinder [31].

In silico comparative analysis of the pSEOhiM1593a plasmid and Enterobacteriaceae-derived plasmids

The genetic features of pSEOhiM1593a (e.g., antimicrobial resistance genes, plasmid replicon types, and ISs) were compared with those of plasmids derived from Enterobacteriaceae and *Salmonella* (Supplementary Table 3). The plasmids used for this comparison were identified by BLASTN searches based on their similarities (coverage >99%) with pSEOhiM1593a. The nucleotide sequence data for these plasmids were obtained from GenBank (accession nos. CP009564, MH760469, HQ023864, KP056256, KR559888, MF344573, and KJ909290). All sequence data from the plasmids were reanalyzed using PlasmidFinder and ResFinder [1, 12], and ISs in the plasmids were confirmed using ISfinder [31].

Ethics statement

This study was authorized by the Ethics Regulations Related to Epidemiological Research at the Fukuoka Institute of Health and Environmental Sciences (permission number R2-4) and its guidelines were observed.

Data availability

The complete, annotated genomic sequences from the *S.* Ohio and *S.* Cerro strains are deposited in the DDBJ under accession nos. AP024347–AP024353 and AP024345–AP024346, respectively. The obtained short- and long-read sequence data are deposited in the DDBJ as follows: *S.* Ohio (SEOhiM1593; BioProject PRJDB10937, BioSample SAMD00206786, DRA accession nos. DRR261749 and DRR261750); *S.* Ohio (SEOhiM1960; BioProject PRJDB10937, BioSample SAMD00206787, DRA accession nos. DRR261751 and DRR261752); *S.* Ohio (SEOhiM2008; BioProject PRJDB10937, BioSample SAMD00206788, DRA accession nos. DRR261753 and DRR261754); and *S.* Cerro (SECerM2017; BioProject PRJDB10937, BioSample SAMD00264093, DRA accession nos. DRR261747 and DRR261748).

RESULTS

Salmonella isolates and serotypes

Altogether, 224 *Salmonella* isolates were obtained from 224 samples. The 224 isolates were recovered from the 224 samples originating from one breeder farm (n=4), two hatcheries (n=2), five pullet-rearing farms (n=20), 39 layer farms (n=194), and two EGPs (n=4) (Table 1). The 224 *Salmonella* isolates derived from pooled eggshell samples and environmental egg production samples from the LB-EP chains were classified as the 36 serotypes listed in Supplementary Table 4.

Table 2. Antimicrobial resistance patterns of *Salmonella* isolates and *bla* gene detection

Serotypes	Antimicrobial resistance patterns[a]	*bla* genes	Phenotypic tests	No. of isolates by sample source						Total No. of isolates
				Eggshell	Environment					
					EGP[b]	Layer farm	Pullet-rearing farm	Hatchery	Breeder farm	
Braenderup (n=36)	ABPC-CPDX	bla_{CMY-2}	AmpC[c]			1				1
	SM			1						1
	No resistance			23	3	8				34
Ohio (n=22)	ABPC-CPDX-CP-SM-TC	bla_{CMY-2}	AmpC			1	11			12
	SM-CP-TC					1				1
	No resistance					9				9
O7:b:− (n=1)	ABPC-CPDX-CP-SM-TC	bla_{CMY-2}	AmpC				1			1
Cerro (n=20)	ABPC-CPDX	$bla_{CTX-M-14}$	ESBL[d]	1						1
	SM			3						3
	No resistance			6		7		1	2	16
Infantis (n=22)	ABPC-KM-NA-SM-TC	bla_{TEM-1}	NP[e]					1		1
	SM-CP			1						1
	SM			1						1
	No resistance			14		5				19
Kentucky (n=4)	ABPC-CPFX-NA-SM-TC	bla_{TEM-1}	NP			2				2
	ABPC-CPFX-NA	bla_{TEM-1}	NP			2				2
Agona (n=9)	TC					6				6
	No resistance					3				3
Alachua (n=21)	SM					1	1			2
	No resistance			5		14				19
Livingstone (n=4)	SM-CP					1				1
	No resistance			1		2				3
Manhattan (n=2)	SM-TC					1	1			2
Schwarzengrund (n=1)	SM-NA-TC			1						1
Tenessee (n=2)	SM					1				1
	No resistance			1						1
Others[f] (n=80)	No resistance			30	1	41	6		2	80

[a] ABPC: ampicillin, CP: chloramphenicol, CPFX: ciprofloxacin, CPDX: cefpodoxime, KM: kanamycin, NA: nalidixic acid, SM: streptomycin, TC: tetracycline. [b] EGP: egg shell grading and packing centers. [c] Isolates confirmed as AmpC β-lactamase producers. [d] An isolate confirmed as an extended-spectrum β-lactamase (ESBL) producer. [e] NP: these isolates produced neither ESBL nor AmpC β-lactamase. [f] Including the 24 serotypes listed in Supplementary Table 4.

Antimicrobial susceptibility tests and bla gene determination

The antimicrobial susceptibility tests showed that 40/224 *Salmonella* isolates were resistant to at least one antimicrobial (Table 2). In fact, 184 were susceptible to all 10 of the tested antimicrobials. None of the isolates (n=4) from a breeder farm displayed antimicrobial resistance, but one of the two isolates from the hatcheries did. The 40 antimicrobial-resistant *Salmonella* isolates generated 11 antimicrobial resistance patterns, with ABPC-CPDX-CP-SM-TC being the most frequently observed pattern (n=13). Among these 40 isolates, 30, 26, 20, 16, 15, 6, 4, and 1 were resistant to SM, TC, ABPC, CP, CPDX, NA, CPFX, and KM, respectively (Supplementary Table 5). No isolates were resistant to AZM or MEPM. Of the 20 isolates resistant to ABPC, 14 were AmpC β-lactamase-producers (*S.* Ohio; n=12, *S.* Braenderup; n=1, O7:b:- ; n=1) and carried bla_{CMY-2}; one of the 20 isolates was an ESBL-producer (*S.* Cerro; n=1) and carried $bla_{CTX-M-14}$ (Table 2). Five (*S.* Kentucky; n=4, *S.* Infantis; n=1) of the 20 isolates did not carry a *bla* gene other than the bla_{TEM-1} non-ESBL gene. The MICs of the ESCs from the four *Salmonella* strains (SEOhiM1593, 1960 and 2008, and SECerM2017) are shown in Supplementary Table 6. No significant differences were observed in the MICs of the ESCs between strain SEOhiM1960, which harbors multiple bla_{CMY-2} genes, and the other strains with a single bla_{CMY-2}.

Epidemiological information for the ESC-resistant strains carrying bla_{CMY-2} or $bla_{CTX-M-14}$

ESC-resistant *Salmonella* strains harboring bla_{CMY-2} (n=14 in total: *S.* Ohio, n=12; *S.* Braenderup, n=1; untypeable with O7:b:-, n=1) and $bla_{CTX-M-14}$ (*S.* Cerro, n=1) were isolated from two or three pullet-rearing farms and a layer farm belonging to egg-production company A in prefecture A, a layer farm belonging to company B in prefecture B, and a layer farm belonging to company Q in prefecture C (Supplementary Table 2). All three prefectures are contiguously located. One *S.* Cerro strain carrying the $bla_{CTX-M-14}$ (SECerM2017) obtained from a layer farm belonging to company Q and three *S.* Ohio strains carrying bla_{CMY-2}

Table 3. Chromosome and plasmid analysis on *Salmonella enterica* harboring bla_{CMY-2} or $bla_{CTX-M-14}$ from whole genome sequencing

Strain name	Serotype	DNA type	Plasmid name	*bla* gene	Other antimicrobial resistant genes	No. of IS*26* elements	No. of IS*Ecp1* elements within the region flanking the *bla* gene[a]	Replicon type	Size (bp)	Strain origin			
										Facility type	Sample type	Facility name	Isolation date
SEOhiM1593	*S.* Ohio[b]	Chromosome	-	ND[c]	*aac(6')-Iaa*	0	0	-	4,867,732	Pullet-rearing farm	Environment	a1	January 2010
		Plasmid	pSEOhiM1593a	bla_{CMY-2}	*aph(3'')-Ib*, *aph(6)-Id*, *floR*, *sul2*, *tet(A)*	3	1	IncA/C_2	92,251				
SEOhiM2008	*S.* Ohio	Chromosome	-	bla_{CMY-2}	*aph(3'')-Ib*, *aph(6)-Id*, *floR*, *sul2*, *tet(A)*	4	1	IncA/C_2[d]	4,902,159	Pullet-rearing farm	Environment	a1	June 2012
		Plasmid	pSEOhiM2008a	ND	ND	0	0	Col440I	2,264				
SEOhiM1960	*S.* Ohio	Chromosome	-	bla_{CMY-2}	*aac(6')-Iaa*	0	1	-	4,872,199	Layer farm	Eggshell	a5	January 2012
		Plasmid	pSEOhiM1960a	bla_{CMY-2}	*aph(3'')-Ib*, *aph(6)-Id*, *floR*, *sul2*, *tet(A)*	3	1	IncA/C_2	92,095				
		Plasmid	pSEOhiM1960b	ND	ND	0	0	Col440I	2,264				
SECerM2017	*S.* Cerro	Chromosome	-	$bla_{CTX-M-14}$	ND	0	1	-	4,479,959	Layer farm	Eggshell	q1	August 2012
		Plasmid	pSECerM2017a	ND	ND	0	0	Untypeable	2,105				

[a] Number of IS*Ecp1* elements within 5,000 bp of the *bla* gene. [b] *Salmonella enterica* subsp. *enterica* serovar Ohio. [c] ND: not-detected. [d] The integration region in this strain is structured almost identically to the IncA/C_2 plasmids in the SEOhiM1593 and SEOhiM1960 strains.

(SEOhiM1593, 1960, and 2008) obtained from a pullet-rearing farm and a layer farm belonging to company A, were further analyzed as described below.

WGS identification of antimicrobial resistance genes, plasmid replicon types, and ISs

Table 3 shows the results of the analysis on antimicrobial resistance genes, plasmid replicon types, and ISs from the three *S.* Ohio strains (SEOhiM1593, 1960, and 2008) and the *S.* Cerro strain (SECerM2017). Based on this analysis, we classified pSEOhiM1593a and 1960a as IncA/C_2 plasmids. The two IncA/C_2 plasmids exhibit high-level nucleotide sequence identity with each other (Fig. 1A). Moreover, nucleotide sequences on the SEOhiM2008 chromosome were assigned as belonging to an IncA/C_2 plasmid (Table 3). The chromosome region of SEOhiM2008 and the two IncA/C_2 plasmids exhibit high nucleotide sequence identity with each other (Fig. 1A and 1C). Therefore, the evidence supports IncA/C_2 plasmid integration into the SEOhiM2008 strain's chromosome (Fig. 1D). The chromosomal region encompassing nucleotide position 1,090,811–1,183,061 surrounded by IS*26* shares high sequence identity with the corresponding region of the plasmid belonging to pSEOhiM1593a (nucleotide position 1–92,251), except for an inverted region sandwiched (2,064 bp) between two IS*26* elements. This chromosomal region is flanked by a target site duplication of 8 bp (5′-AAGAATAT-3′) suggesting that IS*26* has integrated this region. pSEOhiM1593a, pSEOhiM1960a, and the chromosomal region of strain SEOhiM2008 assigned as belonging to an IncA/C_2 plasmid harbored genes conferring resistance against aminoglycoside (*aph(3")-Ib* and *aph(6)-Ib*), florfenicol/chloramphenicol (*floR*), sulfonamide (*sul2*), and tetracycline (*tet(A)*), as well as ESC (bla_{CMY-2}). Strains SEOhiM1593, SEOhiM1960, and SEOhiM2008 were isolated from different production levels (pullet-rearing farm and layer farm strains) in the same company (Supplementary Table 2). bla_{CMY-2} was detected on the SEOhiM1960 strain's chromosome (Table 3 and Fig. 1B). Furthermore, the chromosomal region of the nucleotide position between 2,412,761 and 2,415,912 is identical to the partial region carrying bla_{CMY-2} along with IS*Ecp1* in pSEOhiM1593a (nucleotide position 57,825–60,976).

Of note, $bla_{CTX-M-14}$ is present on the SECerM2017 chromosome. We found that the region carrying $bla_{CTX-M-14}$ along with IS*Ecp1* on this chromosome (3,006 bp, nucleotide position 1,581,874–1,584,879) is identical to the corresponding region in the p14E509-CTXM plasmid (nucleotide position 20,608–23,613) from a Chinese clinical *E. coli* strain in 2014 (GenBank accession no. MG764547.1) (Fig. 2).

In silico comparative analysis on the pSEOhiM1593a plasmid and Enterobacteriaceae-derived plasmids

We compared the genetic features of pSEOhiM1593a with those of Enterobacteriaceae family plasmids, including *Salmonella* from other studies (Supplementary Table 3). The plasmids used for the comparison were found in Enterobacteriaceae derived from humans and animals. All the plasmids were found to carry multiple antimicrobial resistance genes and more than one IS*26*. We identified antimicrobial resistance genes (bla_{CMY-2}, *aph(3")-Ib*, *aph(6)-Id*, *floR*, *sul2*, and *tet(A)*) sharing among all the plasmids, but the pKP-Gr642 plasmid carries bla_{CMY-4} instead of bla_{CMY-2}.

DISCUSSION

Our study has three main findings. First, *Salmonella* harboring bla_{CMY-2} (*S.* Ohio; n=12, *S.* Braenderup; n=1, untypeable with O7:b:-; n=1) and harboring $bla_{CTX-M-14}$ (*S.* Cerro; n=1) were found in LB-EP chains in Japan. Second, two *S.* Ohio strains, SEOhiM1593 and 1960, harbor IncA/C_2 plasmids with multiple antimicrobial resistance genes including bla_{CMY-2}, IS*Ecp1*, and IS*26*. Third, strain SEOhiM1960 possesses a partial region of an IncA/C_2 plasmid on its chromosome and strain SEOhiM2008 possesses the entire region of an IncA/C_2 plasmid on its chromosome, and these chromosomal integrations involved IS*Ecp1* or IS*26*, respectively. Despite numerous reports of ESC-resistant *Salmonella* in broiler breeding chains [3, 6, 28], few studies have investigated it in any great depth in LB-EP chains. *S.* Virchow harboring $bla_{CTX-M-9}$, *S.* Infantis harboring $bla_{CTX-M-65}$, and *S.* Gallinarum harboring bla_{TEM} were reported in LB-EP chains in Spain, Ecuador, and India, respectively [24–26]. Herein, we found that ESC-resistant *Salmonella* is present in Japanese LB-EP chains (Table 2 and Supplementary Table 2). Furthermore, because pSEOhiM1593a, pSEOhiM1960a, and the chromosomally integrated pSEOhiM1593a-related plasmid in strain SEOhiM2008, whose replicon type is classified as IncA/C_2, were found in the upper- and lower-level strains (SEOhiM1593, 1960, and 2008), this indicates that antimicrobial-resistance gene transmission through IncA/C_2 plasmids occurred among *Salmonella* in the layer chicken breeding chains. Better hygiene management in breeding chains, including the upper levels of layer chicken breeding chains, is needed to control egg contamination with antimicrobial-resistant *Salmonella*.

Obtaining pSEOhiM1593a-related plasmids with IncA/C_2 from Enterobacteriaceae might enhance each recipient's survival in various hosts via the acquisition of antimicrobial resistance genes. IncA/C_2 plasmids carrying bla_{CMY} are disseminated among Enterobacteriaceae from various sources and countries (Supplementary Table 3), and their dissemination might involve a specific antimicrobial resistance gene set. By acquiring IncA/C_2 plasmids with the bla_{CMY-2}, bla_{CMY-4}, *aph(3")-Ib*, *aph(6)-Id*, *floR*, *sul2*, and *tet(A)* antimicrobial-resistance gene set, Enterobacteriaceae will be resistant to β-lactams, aminoglycosides, florfenicol/chloramphenicol, sulfas, and tetracyclines. Harboring IncA/C_2 plasmids containing this gene set would advantage Enterobacteriaceae survival in the presence of the above-named antimicrobials. According to the US Food and Drug Administration's research in 2018, aminoglycosides, sulfas, and tetracyclines were used for infections in swine whereas cephalosporins, penicillins, aminoglycosides, sulfas, and tetracyclines were used for infections in cattle [34]. It is possible that Enterobacteriaceae including *Salmonella* recovered from swine and cattle in the US might have survived by obtaining the IncA/C_2 pCVM21550 and pAR060302 plasmids listed in Supplementary Table 3. Japanese governmental research in 2018 indicated that penicillins, aminoglycosides,

sulfonamides, and tetracyclines were used on layer chickens in Japan [21]. The present study might also show that acquisition of pSEOhiM1593a-related plasmids with IncA/C_2 may benefit the persistence of *S.* Ohio in LB-EP chains. However, no data were available to this study about the use of antimicrobials on these Japanese farms; hence, we were unable to determine whether antimicrobial use was a relevant factor on these farms. Further investigation is required to confirm that IncA/C_2 plasmids harboring the aforementioned antimicrobial resistance gene set might be more likely to spread among Enterobacteriaceae in various hosts and countries that have used antimicrobials.

IS*Ecp1* may spread the *bla* gene among *Salmonella* via the integration of a partial plasmid region, whereas IS*26* may disseminate multiple antimicrobial resistance genes among *Salmonella* through incorporation of the whole plasmid or a broad range of plasmids. Herein, we confirmed that the plasmid-derived partial region containing bla_{CMY-2} or $bla_{CTX-M-14}$ was integrated by IS*Ecp1* into the *S.* Ohio chromosome or *S.* Cerro chromosome, respectively. Furthermore, IS*26* may disseminate multiple antimicrobial resistance genes (including the *bla* gene) within the plasmids present among *Salmonella*. IS*26* reportedly occurred only after a partial regional integration of the IncY and IncA/C_2 fusion plasmid, which carried $bla_{CTX-M-15}$ into the *S.* Concord chromosome [8]. However, IS*26* possibly integrated a large portion (~120 kbp) of an *E. coli* IncA/C plasmid containing multiple antimicrobial resistance genes (including bla_{CMY-2}) into the *S.* Typhimurium chromosome [27]. Our own findings indicate that *S.* Ohio obtained multiple antimicrobial resistance genes (including bla_{CMY-2}) via IS*26*-mediated integration of an intact pSEOhiM1593a-related plasmid. In fact, IS*Ecp1* integrated a partial *bla* gene-carrying region existing in pSEOhiM1593a-related plasmid into *S.* Ohio strain SEOhiM1960 (Fig. 1B), while IS*26* integrated the whole region of the pSEOhiM1593a-related plasmid into *S.* Ohio strain SEOhiM2008 (Fig. 1C), which resulted in the dissemination of multiple antimicrobial resistance genes via IS*26*. Overall, it seems likely that antimicrobial resistance genes, including the *bla* gene, have dispersed in *Salmonella* within the layer breeding chains via ISs-mediated-integration of plasmids.

In conclusion, *Salmonella* obtains numerous antimicrobial resistance genes, especially *bla* genes on the IncA/C_2 plasmid, through partial regional plasmid integration by IS*Ecp1* and the larger or whole regional integration of the plasmid by IS*26*. Antimicrobial resistance genes such as bla_{CMY-2} in *Salmonella* might be transmitted among the upper and the lower levels of layer breeding chains via the IncA/C_2 plasmids containing IS*Ecp1* and IS*26*. Further studies are required to fully understand the IS-mediated-integration of plasmids in *Salmonella* in veterinary medicine.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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