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Identification of a Novel Genomic Island Conferring Resistance to Multiple Aminoglycoside Antibiotics in *Campylobacter coli*

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Historically, the incidence of gentamicin resistance in *Campylobacter* has been very low, but recent studies reported a high prevalence of gentamicin-resistant *Campylobacter* isolated from food-producing animals in China. The reason for the high prevalence was unknown and was addressed in this study. PCR screening identified aminoglycoside resistance genes *aphA-3* and *aphA-7* and the *aadE–sat4–aphA-3* cluster among 41 *Campylobacter* isolates from broiler chickens. Importantly, a novel genomic island carrying multiple aminoglycoside resistance genes was identified in 26 aminoglycoside resistant *Campylobacter coli* strains. Sequence analysis revealed that the genomic island was inserted between *cadF* and *COO1582* on the *C. coli* chromosome and consists of 14 open reading frames (ORFs), including 6 genes (the *aadE–sat4–aphA-3* cluster, *aacA-aphD*, *aac*, and *aadE*) encoding aminoglycoside-modifying enzymes. Analysis by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing indicated that the *C. coli* isolates carrying this unique genomic island were clonal, and the clone of PFGE subtype III and sequence type (ST) 1625 was particularly predominant among the *C. coli* isolates examined, suggesting that clonal expansion may be involved in dissemination of this resistance island. Additionally, we were able to transfer this genomic island from *C. coli* to a *Campylobacter jejuni* strain using natural transformation under laboratory conditions, and the transfer resulted in a drastic increase in aminoglycoside resistance in the recipient strain. These findings identify a previously undescribed genomic island that confers resistance to multiple aminoglycoside antibiotics. Since aminoglycoside antibiotics are used for treating occasional systemic infections caused by *Campylobacter*, the emergence and spread of this antibiotic resistance genomic island represent a potential concern for public health.

Campylobacter is one of the most common food-borne pathogens worldwide. Two thermophilic species, *Campylobacter jejuni* and *Campylobacter coli*, are responsible for most *Campylobacter* infections in humans. Both *C. jejuni* and *C. coli* are prevalent in food-producing animals, and the extensive use of antimicrobials in food animal production has led to an increasing prevalence of antibiotic-resistant *Campylobacter* strains, which can be transmitted to humans via the food chain (1, 3).

Clinical therapy of *Campylobacter*-induced gastroenteritis, when needed, is often conducted with macrolide and fluoroquinolone antibiotics, but severe systemic infections, which occur sporadically, requires the use of an aminoglycoside antibiotic, such as gentamicin (2). Aminoglycosides are highly potent, broad-spectrum, bactericidal antibiotics and are commonly used in the treatment of infections caused by both Gram-negative and Gram-positive organisms in humans (25). Additionally, several aminoglycoside agents, such as neomycin and amikacin, are also used in conventional broiler chicken and swine production in some countries, including China. Among the known mechanisms of aminoglycoside resistance, enzymatic modification is the most important and prevalent one in several bacterial species, including *Campylobacter* spp. (17, 25). Based on the reactions they catalyze, aminoglycoside-modifying enzymes are divided into three groups: aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltranferases (ANTs), and aminoglycoside phosphotransferases (APHs) (17). In *Campylobacter*, each group of aminoglycoside-modifying enzymes has been detected. The 3′,9-aminoglycoside adenyltransferase gene (*aadA*) and 6-aminoglycoside adenyltransferase gene (*aadE*) confer resistance to streptomycin; the *aacA4* gene, encoding an AAC, confers resistance to gentamicin and tobramycin (11, 15), and 3′-APHs, which are the most frequently reported, are responsible for resistance to kanamycin and neomycin (30).

The aminoglycoside resistance genes in *Campylobacter* have been detected on multidrug-resistant plasmids, integrons, and transposons (9, 13). In addition, the aminoglycoside resistance gene cluster, *aadE–sat4–aphA-3*, has been detected on transmissible plasmids in *C. jejuni* but has not been identified in *C. coli* (9, 13). Although kanamycin resistance has been reported in multiple studies, the prevalence of gentamicin-resistant *Campylobacter* is generally low (<1%) in studies from various countries (3, 12, 27). However, two recent studies conducted in China indicated that the frequency of aminoglycoside resistance, especially gentamicin resistance, is high (>20%) in *C. coli* isolated from both broiler chickens and swine (4, 16). The resistance mechanisms and the reason for the high prevalence of gentamicin-resistant *Campylobacter* in China are not known. To answer these questions, we analyzed aminoglycoside-resistant *Campylobacter* isolates derived from chickens

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and identified a unique genetic structure containing multiple aminoglycoside resistance genes, including *aadE–sat4–aphA-3* and *aacA-aphD*, on the chromosome of *C. coli*. This novel chromosomal aminoglycoside resistance gene cluster has not been reported previously and is associated with clonal isolates of *C. coli*, suggesting the possibility of clonal expansion of aminoglycoside-resistant *C. coli* in broiler chickens in China. Furthermore, we demonstrated the potential dissemination of this unique genetic structure from *C. coli* to *C. jejuni* under laboratory conditions. These findings provide new insights into the dissemination of aminoglycoside resistance in *Campylobacter*.

MATERIALS AND METHODS

***Campylobacter* strains and susceptibility testing.** In total, 138 *Campylobacter* isolates (*C. jejuni*, $n = 90$; *C. coli*, $n = 48$), derived from two broiler chicken slaughterhouses located in eastern (Penglai) and western (Shenxian) regions of Shandong province, were used in this study. Detailed information on their susceptibilities to various antimicrobials (including gentamicin) is provided in a previous publication (4). In this study, the isolates were further examined for their susceptibility to kanamycin, neomycin, and amikacin using the agar dilution method, according to the guideline M31-A3 of the Clinical and Laboratory Standards Institute (CLSI) (5). The reference strain *C. jejuni* ATCC 33560 was used as a quality control strain. The breakpoint for kanamycin is 64 μg/ml according to the CLSI (5). Since there are no CLSI breakpoints for neomycin and amikacin in *Campylobacter*, we used the breakpoints of the two agents for *Enterobacteriaceae*, which are 32 μg/ml and 64 μg/ml, respectively (5, 26).

Detection of aminoglycoside resistance genes and molecular analysis. All kanamycin-resistant strains were selected to investigate the known aminoglycoside resistance genes *aphA-1*, *aphA-3*, and *aphA-7* and the *aadE-sat4-aphA-3* gene cluster, which were previously found in *Campylobacter* (9, 14, 22). The primers used to detect the *aphA-3* gene and *aadE-sat4-aphA-3* gene cluster were described previously (9), and the primers designed for the amplification of *aphA-1* (aphA-1-F, 5′-CGTATTTCGT CTCGCTCAG-3′; aphA-1-R, 5′-CCGACTCGTCCAACATCA-3′) and *aphA-7* (aphA-7-F, 5′-ATCCGATAAACTGAAAGTAC-3′; aphA-7-R, 5′-ATAATCCGGTTCAAGTCCC-3′) were based on previously published sequences (14, 22). Genomic DNA was isolated from the strains using a Wizard genomic DNA purification kit (Promega, Madison, WI). Plasmid DNA was extracted using a Qiagen plasmid extraction midikit (Qiagen, Hilden, Germany). Southern blotting was conducted to determine the location of the *aadE–sat4–aphA-3* gene cluster; the primers specific for the gene cluster (CF, 5′-GGATGGATTCCTATGAAAACAT-3′; CR, 5′-GGCTTTGTTCATCTTCATACTCT-3′) were designed according to previously published sequences (9), and the PCR product was used as the probe for hybridization. The probe was nonradioactively labeled with a DIG High Prime I DNA labeling and detection starter kit (Roche Diagnostics, Mannheim, Germany). Hybridization was performed at 42°C for 14 h. Membranes were washed twice at room temperature (22 to 25°C) in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% SDS for 5 min and twice at 68°C in 0.1× SSC, 0.5% SDS for 15 min. DIG was detected with specific antibodies using a DIG High Prime I DNA labeling and detection starter kit (Roche Diagnostics, Mannheim, Germany) according to the recommendations of the supplier. In addition, the flanking regions of the gene cluster were sequenced by a modified random primer sequencing walking strategy as described previously (29).

Pulsed-field gel electrophoresis (PFGE). PFGE was performed using a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA), according to the rapid protocol for *Campylobacter* (18). *Salmonella* H9812 was used as the reference marker (digested with XbaI), while all *Campylobacter* isolates were digested with SmaI. The running conditions were as follows: 0.5× Tris-borate-EDTA–1% SeaKem Gold agarose (FMC BioProducts, Rockland, ME) at 14°C and 6 V/cm for 18 h with switch times ranging from 6.75 s to 38.35 s and an included angle of 120°. The dendrograms were constructed from the PFGE data by UPGMA (unweighted pair group method with arithmetic average) with the Dice coefficient using the InfoQuest FP software version 4.5 (Bio-Rad Laboratories).

Multilocus sequence typing (MLST). MLST of the seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*) for *C. coli* was performed following the method described previously (7). Briefly, all the housekeeping genes were amplified, and the PCR products were run on an agarose gel to confirm the correct amplicon size and then purified using a Tiangen purification midikit following the manufacturer's instructions (Tiangen, Beijing, China). All purified products were sent to the Beijing Genomic Institute (BGI) for sequencing using the forward and reverse primers of these genes. The allelic profiles and the sequence types were generated by carrying out a BLAST search with the *Campylobacter* sequences in the MLST database (http://www.pubmlst.org/campylobacter).

Natural transformation. A transformation assay using Mueller-Hinton agar was performed according to the method described by Wang and Taylor with minor modifications (28). Genomic DNAs purified from aminoglycoside-resistant *C. coli* strains served as the donors, while aminoglycoside-sensitive *C. jejuni* NCTC11168 was used as the recipient. Briefly, fresh recipient cells (20- to 24-h growth on Mueller-Hinton [MH] agar, grown microaerobically at 42°C) were spread on MH agar at about 5×10^7 cells per plate (in triplicate) and incubated for 6 h (microaerobically at 42°C). Subsequently, 1 μg of genomic DNA of the donor strain was spotted directly onto the inoculated agar, without additional spreading, and incubation was continued for 5 h at 42°C under microaerobic conditions. The cells were harvested from the MH agar and plated on a selective MH agar plate containing kanamycin (60 μg/ml) (KMHA), which was further incubated for 48 to 72 h at 42°C under microaerobic conditions. The recipient (without donor DNA) inoculated on the same MH agar plate served as a negative control. Transformants on the KMHA plates were selected and confirmed by PCR. The stability of aminoglycoside resistance in the transformants was investigated using the method reported by Kim et al. (10).

Nucleotide sequence accession number. The sequence of the novel gene cluster described in this paper has been submitted to GenBank under accession number JQ655275.

RESULTS

Characterization of aminoglycoside resistance in *Campylobacter*. Among the 138 *Campylobacter* isolates tested (*C. jejuni*, $n = 90$; *C. coli*, $n = 48$), 41 (29.7%, including 5 C. *jejuni* and 36 *C. coli*) were resistant to both kanamycin (MIC ≥ 256 μg/ml) and neomycin (MIC ≥ 32 μg/ml), and 36 (26%, including 2 *C. jejuni* and 34 *C. coli*) were resistant to amikacin (MIC ≥ 64 μg/ml). All 36 amikacin-resistant isolates were also resistant to both kanamycin and neomycin. The prevalence of resistance to the tested aminoglycosides was significantly higher in *C. coli* (>71%) than in *C. jejuni* (<5.6%) ($P < 0.01$). Over half of the kanamycin-resistant isolates (65.9%, 26/41) harbored the *aadE–sat4–aphA-3* cluster, and all of them were *C. coli*. In addition, all these *aadE–sat4–aphA-3*-carrying *C. coli* isolates were resistant to gentamicin. The other 15 kanamycin-resistant isolates harbored only either *aphA-7* or *aphA-3* and were not resistant to gentamicin. The *aphA-7* gene was present only in the *Campylobacter* isolates from Shenxian, including 2 *C. jejuni* and 10 *C. coli* isolates. The single *aphA-3* gene was observed only in three *C. jejuni* strains, one from Shenxian and two from Penglai. No *aphA-1* gene was detected among the kanamycin-resistant isolates. The distribution of kanamycin resistance genes in *Campylobacter* is summarized in Table 1.

Genotyping. To facilitate understanding of the transmission of the aminoglycoside resistance genes, the genetic diversity of the 36 aminoglycoside-resistant *C. coli* isolates that harbored *aphA7* or

TABLE 1 Distribution of kanamycin resistance determinants in Km^r isolates detected by PCR

Kanamycin resistance gene(s)	Species	No. of Km^r isolates: SX (n = 18)	MH (n = 23)
aphA-1	*C. jejuni*	0	0
	C. coli	0	0
aphA-3[a]	*C. jejuni*	1	2
	C. coli	0	0
aphA-7	*C. jejuni*	2	0
	C. coli	10	0
aadE–sat4–aphA-3	*C. jejuni*	0	0
	C. coli	5	21

[a] The isolates harbored only *aphA-3* (strains harboring *aphA-3* as part of *aadE-sat4-aphA3* were not included in this category).

the *aadE–sat4–aphA-3* cluster was determined by PFGE using SmaI digestion (Fig. 1). Using a cutoff of 90% genetic similarity, the isolates were clustered into 5 PFGE subtypes, with subtype III accounting for the majority (21/36) of the isolates. Subtypes I, II, and III harbored the *aadE–sat4–aphA-3* cluster (n = 26), while subtypes IV and V carried the *aphA7* gene. The high genetic similarity (>95%) of the 21 isolates (subtype III) (Fig. 1) that all originated from Penglai (MH strains) suggested that these isolates might have originated from a single clone. However, the isolates from Shenxian (SX strains) exhibited four different PFGE subtypes (I, II, IV, and V).

Twelve aminoglycoside-resistant isolates representative of all the PFGE subtypes and four aminoglycoside-susceptible isolates (SX71, SX73, MH16, and MH25) were selected for MLST typing. The results identified six sequence types (STs) (Table 2). Four STs were identified for the aminoglycoside resistant isolates: ST 5604 (SX30, SX108 and SX114 in PFGE subtype I), ST 1586 (SX81 in PFGE subtype II), ST 1625 (MH4, MH34, MH57 and MH62 in PFGE subtype III), and ST 5510 (SX94, SX117, and SX58 in PFGE subtype IV; SX1 in PFGE subtype V). ST 5604 represents a newly identified ST and is first reported in this study. In general, each PFGE subtype corresponds to a single ST, indicating the consistency of the two typing methods. Notably, all of the typed aminoglycoside resistant isolates belong to clonal complex 828 (Table 2). Two

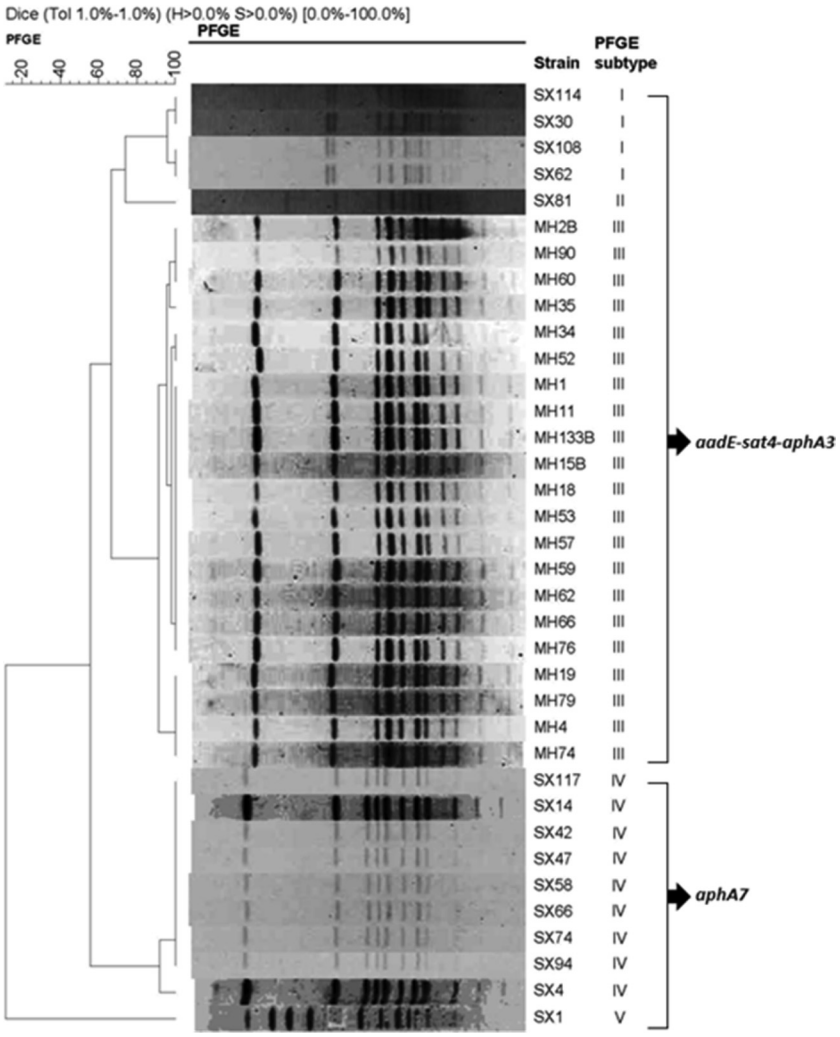

FIG 1 PFGE-based dendrogram for 36 kanamycin-resistant *C. coli* strains harboring the *aphA-7* gene or the *aadE–sat4–aphA-3* gene cluster.

TABLE 2 Aminoglycoside susceptibility, ST, clonal complex, and PFGE pattern of *C. coli* strains from broiler chickens

Aminoglycoside susceptibility	Strains	ST	Clonal complex	PFGE pattern
Resistant	SX114	5604	828	I
	SX30	5604	828	I
	SX108	5604	828	I
	SX81	1586	828	II
	MH57	1625	828	III
	MH62	1625	828	III
	MH34	1625	828	III
	MH4	1625	828	III
	SX94	5510	828	IV
	SX117	5510	828	IV
	SX58	5510	828	IV
	SX1	5510	828	IV
Susceptible	SX71	4248	—[a]	ND[b]
	SX73	4248	—	ND
	MH16	4268	—	ND
	MH25	4268	—	ND

[a] —, not assigned.
[b] ND, not determined.

STs were found for the typed aminoglycoside-susceptible isolates, including ST 4248 (SX71 and SX73) and ST 4268 (MH16 and MH25), whose clonal complexes have not been assigned.

Identification of a novel aminoglycoside resistance gene island. Three isolates harboring the *aadE–sat4–aphA-3* gene cluster (SX81, MH57, and SX114), which belonged to three different PFGE subtypes (Fig. 1) and three different ST types (Table 2), were selected for further genetic analysis. No plasmids were obtained from these isolates by extraction using the Qiagen plasmid extraction midikit and the Wizard Plus SV miniprep purification system (Promega), suggesting that the resistance genes were not carried by plasmids. Southern blotting of PFGE-separated DNA of all tested strains indicated that the *aadE–sat4–aphA-3* cluster was located on large chromosomal DNA fragments digested by SmaI (Fig. 2). The surrounding genetic environment of the *aadE–sat4–aphA-3* cluster in SX81 was sequenced by a modified random primer walking strategy, starting from the *aadE* gene and the *aphA-3* gene. A 15.3-kb segment of the chromosome was sequenced and was found to contain 16 open reading frames (ORFs), including the *aadE–sat4–aphA-3* cluster (Fig. 3; Table 3). A unique segment of 14 ORFs, including several antimicrobial resistance genes [six genes encoding aminoglycoside-modifying enzymes and a truncated *tet*(O) gene], was inserted between the *cadF* gene and the *CCO1582* gene in the chromosomal DNA of *C. coli* SX81. In the sequenced genome of *C. coli* RM2228 (7), the *CCO1583* gene is located between *cadF* and *CCO1582*. A similar genetic organization was also observed in *C. jejuni* (Fig. 3). However, the gene *CCO1583* (or *cj1477c* in *C. jejuni*) was replaced by the 14-ORF segment in *C. coli* SX81. Additionally, the G+C content for this 14-gene cluster is 37.3%, which is significantly higher than that (31.4%) of the entire genome of *C. coli* RM2228. These results indicate that *C. coli* SX81 has acquired a genomic island of 14 ORFs inserted between *cadF* and *COO1582* on the chromosome.

The annotation of the 14-gene island, including the flanking genes *cadF* and *CCO1582*, is shown in Table 3. Among the six genes encoding aminoglycoside-modifying enzymes in this cluster, *sat4* and *aphA-3* in the *aadE–sat4–aphA-3* cluster show 100% nucleotide identity with their homologs in pCG8245 (13), a multidrug-resistant plasmid identified in *C. jejuni*. The *aadE* gene in the *aadE–sat4–aphA-3* cluster of SX81 is 702 bp, encoding a full-length protein that is 97% identical (amino acids) to the homolog in *Enterococcus faecalis*. However, the *aadE* homolog in pCG8245 was truncated (only 516 bp long) and was not functional (13). The *aac* gene located immediately downstream of *cadF* in SX81 showed only 47.2% nucleotide identity to the *aac* gene in pCG8245, although they have the same length (13). The *aac* gene in SX81 encodes an acetyltransferase enzyme that shows 100% amino acids identity to its homolog in *E. faecalis* T2. The *aacA-aphD* gene encodes a protein of 479 amino acids that has 100% identity to the bifunctional aminoglycoside-modifying enzyme AacA-AphD in *Staphylococcus aureus*. However, the *aacA* (*aphD*) gene in SX81 exhibits only 47.7% nucleotide identity to the homolog in pCG8245. Furthermore, the *aacA-aphD* gene in pCG8245 was truncated and encodes only 281 amino acids (13).

To determine whether this unique genomic island exists in the other two *aadE–sat4–aphA-3* cluster-containing *C. coli* strains (SX114 and MH57), a long-range PCR assay was conducted using a Premix LA *Taq* version 2.0 kit (TaKaRa, Dalian, China) and primers cadf-F (5′-GCTCAAGCAATGACACTAAAG-3′) and pfo-R (5′-GGAGAACTAGGTGTAATAGGAT-3′), which are located in the conserved regions of the *cadF* and *COO1582* genes, respectively. An amplicon of 10.6 kb was obtained from both SX114 and MH57 (Fig. 4), and sequencing results showed that it was identical to that in SX81 (data not shown). Furthermore, this unique structure was also detected in the other 23 *aadE–sat4–aphA-3* cluster-harboring *C. coli* isolates using the above-mentioned PCR assays (data not shown). In contrast, the examined aminoglycoside susceptible isolates SX71, SX73, and MH16 did not yield this 10.6-kb fragment but showed a 908-bp fragment

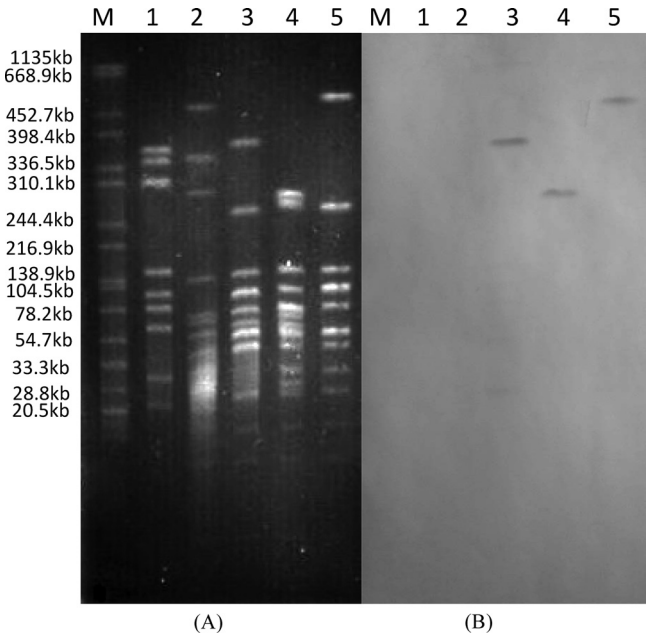

FIG 2 (A) PFGE plugs of kanamycin-resistant *C. coli* SX81, SX114, and MH57 digested with SmaI; (B) Southern hybridization of the *aadE–sat4–aphA-3* cluster to genomic DNA of these isolates. Lane 1, NCTC11168; lane 2, ATCC 33559; lane 3, SX81; lane 4, SX114; lane 5, MH57; lane M, DNA standards.

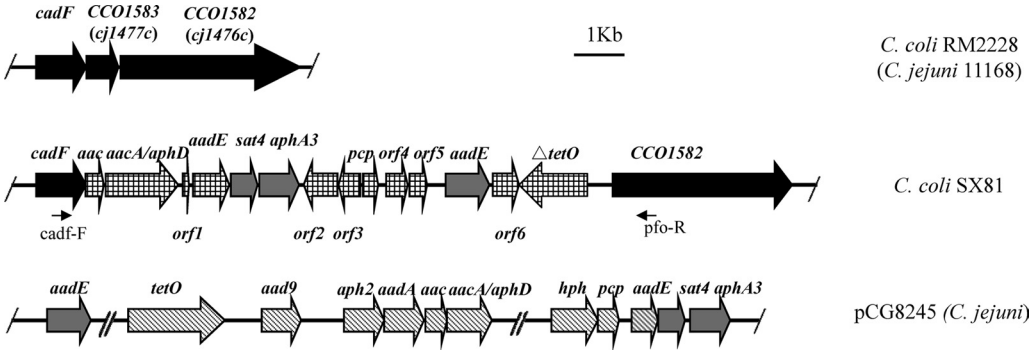

FIG 3 Genomic organization of the aminoglycoside resistance island in *C. coli* SX81 in comparison with plasmid pCG8245 and chromosomal genes of *C. coli* RM2228. Arrows indicate the positions and directions of transcription of the genes. Homologous genes in the three fragments are displayed with the same types of shading. The locations of primers cadf-F and pfo-R used to detect the unique genomic island are indicated.

(Fig. 4), which was determined to be the *CCO1583* gene by sequencing. Additionally, PCR testing of the kanamycin-resistant isolates that harbored only *aphA-7* or *aphA-3* did not identify the 14-gene cluster.

Transfer of aminoglycoside resistance via natural transformation. *Campylobacter* is naturally transformable by taking up DNA from the environment. As the unique aminoglycoside-resistant genomic island is inserted into the chromosome of *C. coli* and is not associated with a plasmid or transposable element, we examined if the gene cluster can be transferred to *C. jejuni* by natural transformation under laboratory conditions. The genomic DNAs of the three *C. coli* strains (SX81, MH57, and SX114) were used as the donor DNA, and C. *jejuni* NCTC11168 was used as the recipient strain. With each donor DNA, aminoglycoside resistant transformants were obtained. A 10.6-kb fragment was amplified using the primers cadf-F and pfo-R from the transformants

TABLE 3 Annotation of genes in the genomic island flanked by *cadF* and *cco1582* in *C. coli* SX81

Gene or ORF	Coding region (bp)	G+C content (%)	Length (aa)[a]	Length (aa)[b]	% Identity/% positive (no. of aa)	Annotation and/or accession no.
cadF	121–1119	34.5	332	332	99/99 (332)	Fibronectin-binding protein, *Campylobacter coli* JV20; ZP_07401432
aac	1133–1498	24.9	121	121	100/100 (121)	Acetyltransferase, *Enterococcus faecalis* T2; ZP_05426921
aacA-aphD	1499–2938	24.7	479	479	100/100 (479)	Bifunctional aminoglycoside modifying enzyme AacA-AphD, *Staphylococcus aureus*; NP_863643
orf1	2996–3112	19.7		38		Unknown (no significant match in database)
aadE	3204–3902	37.4	233	225	97/97 (225)	Streptomycin aminoglycoside 6-adenyltransferase, *Enterococcus faecalis* D6; EEU82268
sat4	3914–4444	37.9	176	176	100/100 (176)	Streptothricinacetyltransferase, *Enterococcus faecium*; ZP_05674679.1
aphA-3	4537–5331	44.9	264	264	100/100 (264)	Aminoglycoside phosphotransferase type III, *Enterococcus faecalis*; YP_783930
orf2	5439–6105	44.4	224	199	80/82(199)	Hypothetical protein BSEG_01714, *Bacteroides dorei* 5_1_36, D4; EEO45573
orf3	6127–6550	41.3	150	141	99/99 (141)	Hypothetical protein BSEG_01715, *Bacteroides dorei*5_1_36, D4; EEO45574
pcp	6620–6913	51.2	139	97	100/100 (97)	Pyrrolidone carboxylate peptidase, *Bacteroides* sp. 9_1_42FAA; ZP_04541315
orf4	7067–7495	31.7	132	120	95/98 (120)	Hypothetical protein, *Streptococcus suis*; CBZ42064
orf5	7527–7871	42.0	181	104	98/100 (104)	Unknown, *Campylobacter jejuni*; AAW34157
aadE	8249–9115	35.4	288	288	99/99 (288)	Putative adenyltransferase, *Pediococcus acidilactici*; YP_001965484
orf6	9203–9720	58.9	196	172	88/89(172)	Hypothetical protein CLONEX_02761, *Clostridium nexile* DSM 1787; EEA81276
Δ*tet*(O)	9735–11096	39.2	639	449	100/100 (449)	Tetracycline resistance protein, *Campylobacter coli* RM2228; ZP_00371083
CCO1582	11568–15128	35.3	1186	1186	99/99 (1186)	Pyruvate ferredoxin/flavodoxin oxidoreductase family protein, *Campylobacter coli* RM2228; ZP_00368185

[a] Size of the ortholog protein. aa, amino acids.
[b] Length of the SX81 peptide.

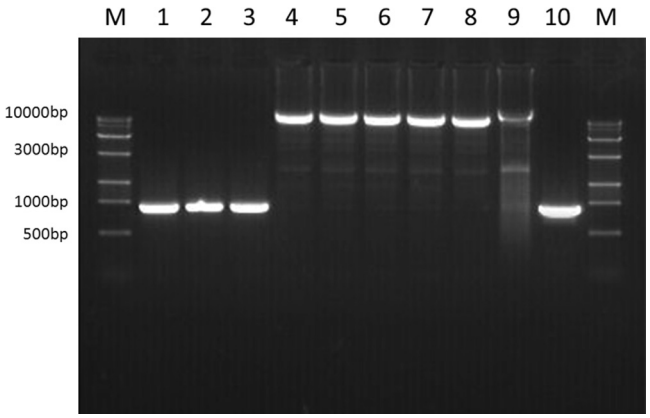

FIG 4 PCR detection of the region between *cadF* and *COO1582* in various strains. Lanes 1 to 3, PCR products from kanamycin-susceptible isolates SX71, SX73, and MH16, respectively; lanes 4 to 6, PCR products from aminoglycoside-resistant strains SX81, SX114, and MH57, respectively; lanes 7 to 9, PCR products from the *C. jejuni* transformants of SX81, SX114, and MH57, respectively; lane 10, PCR products from *C. jejuni* NCTC11168.

(Fig. 4), and DNA sequencing confirmed that the PCR products contained the unique 14-gene cluster, indicating that this genomic island is transferable to *C. jejuni* 11168 by natural transformation. The MICs of streptomycin, kanamycin, gentamicin, neomycin, and amikacin in the recipient, donors, and transformants are summarized in Table 4. Compared to the recipient strain, the transformants showed drastically increased MICs of the five tested aminoglycosides, which were comparable to the MICs in the donor strains. This result suggests that once transferred to *C. jejuni*, the 14-gene cluster confer a high-level resistance to aminoglycoside antibiotics. Additionally, passage of the transformants in antibiotic-free medium indicated that the acquired resistance and the 14-gene cluster remained stable after 14 passages (data not shown).

DISCUSSION

In the present study, a previously undescribed genomic island containing 14 ORFs and conferring aminoglycoside resistance was identified in *C. coli* isolates derived from broiler chickens. This island is inserted between *cadF* and *COO1582* on the *C. coli* chromosome and has a G+C content significantly higher than that of the entire *C. coli* genome. The conclusion that the aminoglycoside resistance island is located on chromosome is based on the following findings. First, no plasmids were isolated from the examined *C. coli* isolates. Second, the resistance island was found to be inserted between chromosomally located genes *cadF* and *CCO1582*, which are highly conserved on *C. coli* and *C. jejuni* genomes and have not been found on any plasmids. Third, the *aadE–sat4–aphA-3* cluster-specific probe hybridized to large SmaI-digested genomic fragments in SX81, SX114, and MH57 (~280 kb, ~370 kb, and ~500 kb, respectively) (Fig. 2). Additionally, this resistance island can be readily transferred to *C. jejuni* via natural transformation, and the genomic location of the transferred resistance genes was further confirmed by long-range PCR (Fig. 4) and sequence analysis. As transfer of large plasmids to *C. jejuni* by natural transformation is quite difficult, its ease of transfer by natural transformation also suggests a chromosomal location of the resistance island. Together, these data indicate that this aminoglycoside resistance gene cluster represents a genomic island inserted into the chromosome of *C. coli*.

This genomic island is unique in several respects. First, it contains genes encoding all known aminoglycoside-inactivating enzymes (AACs, ANTs, and APHs) and thus confers resistance to multiple aminoglycoside antibiotics. Second, it is inserted into the chromosome and is not associated with any known mobile elements or carried by a plasmid, suggesting that transmission is likely mediated by homologous recombination. Third, the genomic island was found in *C. coli* isolates that belong to three clones as defined by PFGE and MLST analysis but was associated with the high prevalence of aminoglycoside-resistant *C. coli* in chickens in China, suggesting that this aminoglycoside resistance island might have undergone spread by both horizontal gene transfer and clonal expansion in the poultry production system in Shandong province of China.

Previously, the gene cluster *aadE–sat4–aphA-3* was detected on plasmids such as pCG8245 in *Campylobacter* (9, 13). In the genomic island identified in this study, all of the aminoglycoside resistance genes encode constitute ORFs and thus are all presumably functional. In contrast, the *aadE* gene in the *aadE–sat4–aphA-3* cluster and the *aacA-aphD* gene in pCG8245 were truncated and not functional (13). *aacA-aphD* is the only identified gene encoding a bifunctional aminoglycoside-modifying enzyme responsible for both gentamicin and kanamycin resistance in *Campylobacter* (13, 19). Identification of this gene in the genomic island explains why the kanamycin-resistant *C. coli* isolates were also resistant to gentamicin. To the best of our knowledge, this is the first report of a chromosomal location of the gene *aacA-aphD* and the gene cluster *aadE–sat4–aphA-3* in *Campylobacter*. In pCG8245, *aadE–sat4–aphA-3* was next to a hybrid of two *H. pylori* transposons, IS*Hp608* and IS*606* (13); however, in the genomic island identified in this study, no transposon-like elements were found between *cadF* and *COOl582*. Based on these observations, we speculate that the entire structure of the aminoglycoside resistance genomic island might be derived from a pCG8245-like plasmid that lost its ability to replicate and was subsequently integrated into the chromosome of *C. coli* by insertion.

TABLE 4 MICs of aminoglycosides in the recipient and donor strains as well as transformants[a]

Antimicrobial agent	MIC (mg/liter) for:									
	NCTC11168	SX81	NT81	$NT^{14}81$	SX114	NT114	$NT^{14}114$	MH57	NT57	$NT^{14}57$
Streptomycin	8	>512	>512	>512	>512	>512	>512	>512	>512	>512
Kanamycin	8	>512	>512	>512	512	512	512	>512	>512	>512
Gentamicin	1	128	256	256	256	256	256	256	256	256
Neomycin	2	32	32	32	64	64	64	32	32	32
Amikacin	4	32	32	32	32	32	32	64	64	64

[a] NT strains are transformants of *C. jejuni* 11168 with donor DNA from SX81 (NT81), SX114 (NT114), and MH57 (NT57). NT^{14} strains are transformants passaged in MH broth in the absence of kanamycin.

Interestingly, all six genes encoding aminoglycoside-modifying enzymes identified in this study appear to have originated from Gram-positive bacteria, including *Enterococcus*, *Staphylococcus*, and *Pediococcus acidilactici*. The *aac*, *aacA-aphD*, *sat4*, and *aphA3* genes showed 100% identity to their corresponding homologs, and the two *aadE* genes also showed more than 97% identity to their Gram-positive homologs (Table 3). This observation is consistent with previous findings by other investigators (6, 21) and further suggests the transfer of antibiotic resistance genes between Gram-positive bacteria and *Campylobacter*. Additionally, several hypothetical ORFs in the genomic island share high homologies with ORFs in *Bacteroides dorei* (Table 3), a Gram-negative organism. Together, these findings suggest that the genes in the genomic island originated from divergent sources.

The *C. coli* isolates harboring the genomic island were from two slaughterhouses located in two geographically separated areas (SX and MH), suggesting the dissemination of *C. coli* carrying this aminoglycoside resistance island in different regions of Shandong province in China. The antibiotic usage records in the two regions indicated that aminoglycoside agents, such as neomycin, amikacin, and gentamicin, were commonly used for curing disease in broiler chickens. This suggests that the emergence and spread of aminoglycoside resistance in *Campylobacter* are likely driven by the selection pressure from antibiotic usage. Based on the genotyping results from PFGE and MLST (Table 2 and Fig. 1), the *C. coli* isolates carrying the antibiotic resistance island can be classified into three different clones. In *Campylobacter*, PFGE and MLST are often used in combination to determine genetic relationship, and isolates of the same PFGE type and ST are regarded as clonal. Clone 1 isolates are PFGE subtype III and ST 1625, while clones 2 and 3 are PFGE subtype I and ST 5604 and PFGE subtype II and ST 1586, respectively. Clone 1 is predominant among the aminoglycoside-resistant isolates examined in this study, suggesting endemic spread of this clone in the broiler production system in Shandong province of China. Furthermore, all three clones belong to clonal complex 828, which was previously identified in *C. coli* isolates from both humans and food-producing animals (23, 24).

The aminoglycoside resistance island identified in *C. coli* in this study is no longer associated with mobile elements, but it might be disseminated via natural transformation mediated homologous recombination. This possibility was demonstrated under laboratory conditions, in which the genes of the genomic island were transferred from *C. coli* to *C. jejuni* and drastically increased aminoglycoside resistance in the recipient *C. jejuni* strain (Table 4). This finding suggests that spread of this aminoglycoside resistance island between *C. coli* and *C. jejuni* could occur in nature, as *Campylobacter* species are naturally transformable. If this occurs, it will pose a concern for public health, as *C. jejuni* is more frequently associated with human campylobacteriosis than *C. coli*. Aminoglycoside antimicrobials, such as gentamicin, are used for treating systemic infections in humans caused by *Campylobacter* (2). Although cases of systemic campylobacteriosis are rare, the clinical consequences can be severe, as the infection may lead to bacteremia, abortion, or even neonatal death (20). Thus, transmission of aminoglycoside-resistant *Campylobacter* represents a concern for food safety and public health.

In conclusion, this study provides the first report of a *C. coli* genomic island that harbors genes conferring resistance to multiple aminoglycoside antibiotics. This unique genomic island consists of 14 ORFs and is disseminated among *C. coli* isolates derived from broiler chickens in China. Horizontal transfer and clonal expansion of the aminoglycoside resistance island were observed, which highlights the need for enhanced efforts to monitor the spread of this genomic island in different *Campylobacter* species.

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