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Role of Cj1211 in Natural Transformation and Transfer of Antibiotic Resistance Determinants in *Campylobacter jejuni*▽†

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***Campylobacter jejuni*, an important food-borne human pathogen, is increasingly resistant to antimicrobials. Natural transformation is considered to be a main mechanism for mediating the transfer of genetic materials encoding antibiotic resistance determinants in *C. jejuni*, but direct evidence for this notion is still lacking. In this study, we determined the role of Cj1211 in natural transformation and in the development of antibiotic resistance in *C. jejuni*. Insertional mutagenesis of Cj1211, a *Helicobacter pylori* ComH3 homolog, abolished natural transformation in *C. jejuni*. In vitro coculture of *C. jejuni* strains carrying either kanamycin or tetracycline resistance markers demonstrated the development of progenies that were resistant to both antibiotics, indicating that the horizontal transfer of antibiotic resistance determinants actively occurs in mixed *Campylobacter* populations. A mutation of Cj1211 or the addition of DNase I in culture media completely inhibited the formation of progenies that were resistant to both antibiotics, indicating that the horizontal transfer of the resistance determinants is mediated by natural transformation. Interestingly, the mutation of Cj1211 also reduced the frequency of emergence of spontaneous mutants that were resistant to fluoroquinolone (FQ) and streptomycin but did not affect the outcome of FQ resistance development under FQ treatment, suggesting that natural transformation does not play a major role in the emergence of FQ-resistant *Campylobacter* strains during treatment with FQ antimicrobials. These results define Cj1211 as a competence factor in *Campylobacter*, prove the role of natural transformation in the horizontal transfer of antibiotic resistance determinants in *Campylobacter*, and provide new insights into the mechanism underlying the development of FQ-resistant *Campylobacter* strains.**

Campylobacter jejuni is a gram-negative organism and a major food-borne human pathogen that causes more than 2 million cases of campylobacteriosis in the United States each year (38). Infection with *Campylobacter* often results in diarrhea, fever, and abdominal cramps. Post-*Campylobacter* infection is also associated with Guillain-Barré syndrome, an acute demyelinating disease of the peripheral nervous system (27, 67). Antibiotic therapy is necessary when *Campylobacter* infections are prolonged or severe or when they occur in immunocompromised hosts. However, *Campylobacter* resistance to various antibiotics is on the rise and has become a concern for public health (20, 44).

Bacteria acquire antibiotic resistance by mutations and horizontal gene transfer (9, 34, 40, 52). Mutations in the genes encoding antibiotic targets help bacteria counteract the attack by antibiotics. For example, point mutations in *gyrA*, *rpoB*, and *rpsL* result in resistance to fluoroquinolone, rifampin, and streptomycin, respectively (34). Horizontal gene transfer is mediated by transduction, conjugation, and natural transformation (40). Transduction is carried out by bacteriophages, while conjugation is mediated by self-replicating plasmids and requires cell-to-cell contact (18, 52). Unlike transduction and conjugation, natural transformation does not require bacteriophages or cell-to-cell contact. Instead, natural transformation involves the uptake of free DNA from the surrounding environment by some competent bacterial species and subsequent homologous recombination to incorporate the internalized DNA into the recipient chromosome (8, 15, 52). Most bacteria possess at least one of the mechanisms for the horizontal transfer of genetic materials (52).

Campylobacter is capable of conjugation and natural transformation (53, 59). Conjugative transfer of antibiotic resistance, often resistance to tetracycline, was reported in *Campylobacter* in culture media and in infected animals (2, 48, 53, 56). Natural transformation is well recognized in *Campylobacter* and has facilitated the genetic manipulation of *Campylobacter* strains for research purposes (24, 59, 60). The natural competence of *Campylobacter* is widely regarded as an important mechanism to generate genetic diversity of this bacterium by mediating horizontal gene transfer (10, 12, 17, 32, 62). It appears that both natural transformation and conjugation potentially play significant roles in the transfer of antibiotic resistance determinants in *Campylobacter*. Bacteriophages infecting different *Campylobacter* strains have been reported (1, 21, 30), but their role in mediating horizontal gene transfer in *Campylobacter* is unknown.

Bacterial competence for natural transformation requires the function of a series of proteins involved in DNA binding, fragmentation, degradation, and transport (8, 14). During the process of natural transformation, the transforming DNA must pass through the hydrophobic membrane barrier to enter the cytosol, and this transmembrane passage is mediated by cyto-

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TABLE 1. *C. jejuni* strains and plasmids used in this study

C. jejuni strain or plasmid	Description	Source or reference
Strains		
NCTC 11168	Wild type	43
JB101	NCTC 11168 derivative; Cj0011c::*cat*	29
JB201	NCTC 11168 derivative; Cj1211::*cat*	This study
JB201C	JB201 harboring pJB1211	This study
JB201P	JB201 harboring pRY108	This study
JB202	NCTC 11168 derivative; *hipO*::*kan*	This study
BQ304	NCTC 11168 derivative; *dcuA*::*tet*(*O*)	Our laboratory
JB203	NCTC 11168 derivative; Cj1211::*cat hipO*::*kan*	This study
JB204	NCTC 11168 derivative; Cj1211::*cat dcuA*::*tet*(*O*)	This study
Plasmids		
pUOA18	*E. coli-Campylobacter* shuttle plasmid; Cm^r	58
pMW10	*E. coli-Campylobacter* shuttle plasmid; Kan^r	63
pRY108	*E. coli-Campylobacter* shuttle plasmid; Kan^r	65
pJB1211	pRY108 derivative; pRY108::Cj1211	This study

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]
Cj1211_F	GCTGCAGGAGTTTTAAGTTCTCAAGGAAAA
Cj1211_R	GAGCAAATCTCCATAGTTTATAAGATGCAA AAATAA
Cat_F	CTAACTTCCGGTGAAGGATATCTAGAAAAA
Cat_R	GCCCATTCTATAGATATATGGATCCGC GCGC
hipO_F	CACAATATGCCTTTTGGTAGCGATAAGAAA
hipO_R	CGCAAGTTTACAGAATTTACAAGAACTT TTTG
Kan_F	CTTATCAATATATCCATGGAATGGGCAAA GCAT
Kan_R	GATAGAACCATGGATAATGCTAAGACAATC ACTAAA
Cj1211_UF	CATCATTTTGCTGCTTTTCTAGATCATT GCTA (XbaI)
Cj1211_UR	AAAAGAGTAAGAAAAAGAATTCCATAGAG ACATTAATAGCCTTTAAAAAAATATTATC TTATGTATG
Cj1211_DF	ATGTCTCTATGGAATTCTTTTTCTTACTC TTTT
Cj1211_DR	GGATTTAGAATTCTTGGATTATTTATCTTTT TGGG (EcoRI)
Cj1211RT_R	GATAATTCCATGGCTCAAAAATTGATTCTT GCTTAAA

[a] Restriction sites embedded in the primers are underlined and indicated in parentheses.

plasmic membrane channel proteins (8, 15). In both gram-negative and gram-positive bacteria, several competence proteins such as *Helicobacter pylori* ComE3, *Neisseria gonorrhoeae* ComA, *Streptococcus pneumoniae* CelB, *Haemophilus influenzae* Rec-2, and *Bacillus subtilis* ComEC have been reported to play a critical role in natural transformation by facilitating the transfer of extracellular DNA to the cytosol (16, 25, 41, 46, 66). In *B. subtilis*, ComEC functions as homodimers in the membrane and forms an aqueous channel through which transforming DNA enters the cytosol (13). In *H. pylori*, the ComE3 protein (a ComEC homolog) is speculated to be a membrane transporter involved in DNA transfer (66). Several competence factors in *C. jejuni* have been identified, including RecA, involved in DNA recombination (23); VirB10, encoded by the pVir plasmid (3, 4); ComEA (Cj0011c), involved in DNA binding (29); and several proteins of the type II secretion pathway (61) and the N-linked protein glycosylation pathway (35). In spite of these advances in our understanding of the natural transformation of *Campylobacter*, little is known about the detailed mechanisms involved in the uptake of DNA through the outer membrane, processing of DNA in the periplasm, and transfer of DNA from the periplasm to the cytosol. Based on the genomic sequence of *C. jejuni* strain NCTC 11168 (43), Cj1211 shares significant amino acid sequence homology (33% identity; E = 5e-34) with *H. pylori* ComE3, a putative membrane channel protein involved in the transfer of DNA (66). However, the role of Cj1211 in the natural transformation of *C. jejuni* is unknown. In addition, the contribution of natural transformation to the horizontal transfer of antibiotic resistance determinants and to the emergence of fluoroquinolone (FQ) resistance in *C. jejuni* has not been formally demonstrated. In this study, we conducted both in vitro and in vivo experiments to address these questions.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. *C. jejuni* NCTC 11168 and its derivatives were used in this study (Table 1). The strains were grown in Mueller-Hinton (MH) medium under microaerobic (5% O_2, 10% CO_2, and 85% N_2) conditions at 42°C. When needed, the culture media were supplemented with kanamycin (50 μg ml^{-1}), tetracycline (5 μg ml^{-1}), or chloramphenicol (10 μg ml^{-1}).

Construction of various mutants. A Cj1211 mutant was constructed by inserting the *cat* gene into the middle of Cj1211. A 1,555-bp fragment containing Cj1211 was PCR amplified with $Vent_R$ DNA polymerase (New England Biolabs, Ipswich, MA) using primers Cj1211_F and Cj1211_R (Table 2). The PCR product was cloned into SmaI-digested pUC19. pUC19 containing Cj1211 was digested with SwaI and ligated with *cat*, which was amplified from pUOA18 (58) with primers Cat_F and Cat_R (Table 2). The orientation of the inserted *cat* gene was confirmed by PCR. The plasmid was electroporated into *C. jejuni* NCTC 11168, and the Cj1211::*cat* mutant was selected on MH agar plates containing chloramphenicol (10 μg ml^{-1}). The Cj1211 mutant was confirmed by PCR and was designated JB201 in this study (Table 1).

To facilitate the measurement of the horizontal transfer of antibiotic resistance determinants in *Campylobacter*, two *C. jejuni* NCTC 11168 mutants harboring different antibiotic markers were constructed. The *hipO* mutant was constructed by the insertion of the *aphA3* gene, encoding kanamycin resistance, into *hipO*. For this purpose, the DNA region containing the *hipO* gene was amplified with Ex Taq (TaKaRa Bio Inc., Japan) using primers hipO_F and hipO_R (Table 2) and cloned into pGEM-T (Promega, Madison, WI). pGEM-T::*hipO* was digested with NheI and blunt ended with Klenow fragment (TaKaRa Bio Inc.). This construct was ligated with *aphA3* DNA, which was amplified with $Vent_R$ DNA polymerase (New England Biolabs) from pMW10 (63) by use of primers Kan_F and Kan_R (Table 2). The resulting plasmid was used as a suicide vector and introduced into *C. jejuni* by electroporation. The transformants were selected on MH agar plates supplemented with kanamycin (30 μg ml^{-1}). The *hipO* mutant was confirmed by PCR and named JB202 (Table 1). *C. jejuni* strain BQ304, in which the *tet*(*O*) gene (encoding tetracycline resistance) was inserted into *dcuA* (B. Guo and Q. Zhang, unpublished data), was also used in this study (Table 1). The *hipO* mutant and the *dcuA* mutant were further used to generate JB203 and JB204 (Table 1). JB203 was generated by the transformation of JB202 using genomic DNA of JB201, and JB204 (Table 1) was

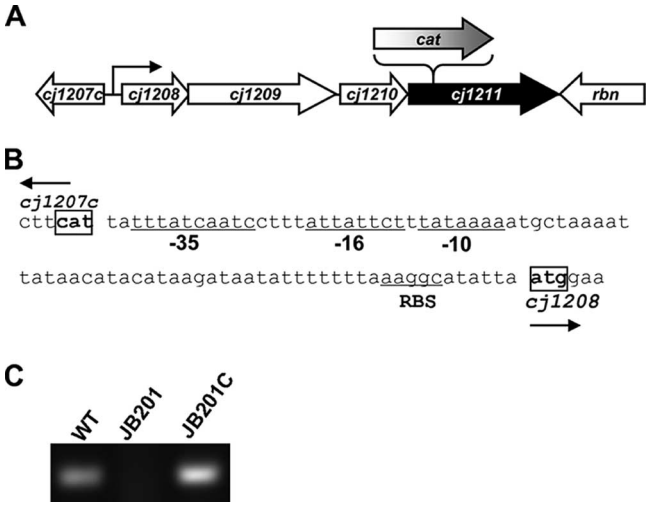

FIG. 1. Transcription unit of Cj1211. (A) Genomic organization of Cj1211 and its flanking genes. Open reading frames and their transcription directions are indicated by boxed arrows. The location of the *cat* insertion in JB201 is indicated with a bracket above Cj1211. The predicted RpoD promoter upstream of Cj1208 is indicated with a curved arrow. (B) Predicted promoter sequences upstream of Cj1208. The −10, −16, and −35 regions and the ribosomal binding site (RBS) are underlined. The start codons of Cj1208 and Cj1207c are boxed. (C) RT-PCR analysis of the transcription of Cj1211 in wild-type NCTC 11168 (WT), JB201, and JB201C.

constructed by transforming BQ304 with the genomic DNA of JB201. Detailed information on these mutants is described in Table 1. The selection of the *hipO* and *dcuA* mutants was based on three criteria. First, the inactivation of these genes must not influence the natural transformation of *C. jejuni*. Second, the growth of one mutant should not suppress the growth of the other in bacterial cocultures. Third, these mutants must carry different antibiotic resistance markers. The *hipO* mutant was previously used for a natural transformation study of *Campylobacter* by other investigators (10). The *dcuA* mutant carried an antibiotic marker different from that of the *hipO* mutant, and JB201 and was readily available in our laboratory. Both the *hipO* and *dcuA* mutants did not affect the natural transformation in *C. jejuni* (see Fig. S1 in the supplemental material) and met the criteria mentioned above.

Complementation of the Cj1211 mutant. Cj1211 and its three upstream genes appear to form an operon (Fig. 1A). There is a predicted promoter in the intergenic region between Cj1207c and Cj1208 (Fig. 1B), and this promoter was fused with Cj1211 for complementation of the Cj1211 mutant. A 173-bp intergenic region between Cj1207c and Cj1208 was amplified with primers Cj1211_UF and Cj1211_UR (Table 2). The entire coding sequence of Cj1211 was PCR amplified with primers Cj1211_DF and Cj1211_DR (Table 2). Primer Cj1211_UR was designed to contain a sequence (33 bp) overlapping that of primer Cj1211_DF (Table 2). After the purification of PCR products with the QIAQuick PCR purification kit (Qiagen, Valencia, CA), these two PCR products were PCR ligated by taking advantage of the overlapped sequence as an annealing region. The ligated product was digested by XbaI and EcoRI and then cloned into pRY108, which is an *Escherichia coli-C. jejuni* shuttle plasmid (65). pRY108 harboring Cj1211 was transferred into JB201 cells by conjugation (39), which resulted in the creation of JB201C (Table 1). As shown in Fig. 1C, the complementation restored the expression of Cj1211 in JB201C cells. RT-PCR was performed with primers Cj1211_DF and Cj1211RT_R (Table 2).

Natural transformation. Natural transformation was done according to a method described previously (59). The donor DNA was isolated from the Cj0011c mutant (designated JB101 in this study) and BQ304 (Table 1). Both mutant strains were readily available in our laboratory, and their antibiotic resistance markers, *aphA3* and *tet*(*O*), confer resistance to kanamycin and tetracycline, respectively, making them suitable for use as donor DNA in transformation experiments. Transformants were selected on MH agar plates containing tetracycline (5 μg ml^{-1}) or kanamycin (30 μg ml^{-1}). The transformation frequency was defined as the number of transformants per 1 μg of DNA divided by the total bacterial number. The transformation experiment with BQ304 DNA was done twice, while the experiment with JB101 DNA was repeated three times. Each transformation experiment was performed in triplicate.

Assay of DNA binding and uptake. DNA binding and uptake were assessed as previously described (61). Briefly, 1.2 μg of chromosomal DNA from *C. jejuni* NCTC 11168 was nick translated using the Roche (Basel, Switzerland) nick translation kit in the presence of 40 μCi [α-^{33}P]dCTP as recommended by the manufacturer. The ^{33}P-labeled DNA was washed by ethanol precipitation and resuspended in TE (10 mM Tris, 1 mM EDTA [pH 8.0]). *C. jejuni* strains were grown on MH agar plates overnight. *C. jejuni* cells were washed three times with MH broth and resuspended to an optical density at 600 nm (OD_{600}) of 0.5. Labeled DNA (100 ng) was added to 1 ml of bacterial suspension. After microaerobic incubation at 37°C for 30 min, half of the bacterial suspension (500 μl) was treated with DNase I (100 μg ml^{-1}) for 10 min at room temperature. The DNase I-treated and nontreated suspensions were washed three times and resuspended in MH broth. Each sample was mixed with 5 ml Ecoscint H liquid scintillation fluid (National Diagnostics, Atlanta, GA) by vigorous shaking for 15 s. Beta emission from ^{33}P was detected by automated liquid scintillation (Beckman Coulter, Fullerton, CA). The radioactivities from DNase I-treated and nontreated samples indicate the levels of DNA uptake and binding, respectively. Triplicate treatments were prepared for each experiment, and the experiment was repeated three times.

DNA exchange in bacterial cocultures. The coculture experiment was done to investigate the role of Cj1211 in mediating DNA transfer during bacterial growth. Cultures of *C. jejuni* grown overnight on MH agar plates were collected and resuspended in fresh MH broth to an OD_{600} of 0.05. For measuring DNA transfer in the Cj1211$^+$ background, where *C. jejuni* contains the intact Cj1211, a bacterial suspension of JB202 (Kan^r) was mixed with an equal volume of BQ304 (Tet^r). The tubes were cultured microaerobically at 37°C with shaking (200 rpm). Samples were taken at 0, 3, 8, and 24 h and plated onto MH agar plates containing kanamycin (30 μg ml^{-1}), tetracycline (5 μg ml^{-1}), or both. To measure DNA transfer in the Cj1211$^-$ background, where Cj1211 was inactivated, equal volumes of JB203 (Kan^r Cm^r) and JB204 (Tet^r Cm^r) were mixed, and the mixture was cultured and sampled as described above. In some experiments, bacterial cultures were washed with MH broth prior to preparation of the mixture to remove free DNA in the inocula. This washing step was done by centrifugation (13,000 × *g* for 1 min) and was repeated five times. The washed bacterial cells were then mixed for the coculture experiment. To determine the involvement of free DNA in the transfer, DNase I was added to the mixed culture at a concentration of 30 μg ml^{-1} in some experiments. The enzyme was added at the beginning of the coculture experiment. Each experiment was performed with three technical replicates and repeated three times.

Frequency of spontaneous mutations in the Cj1211 mutant. To determine the effect of the Cj1211 mutation on the emergence of spontaneous mutants, cultures of JB201 and wild-type NCTC 11168 grown overnight on MH agar plates were resuspended in MH broth to an OD_{600} of 0.07. The bacterial suspensions were incubated at 37°C with shaking (200 rpm) for 4 h ($OD_{600} \approx 0.3$), and the bacterial suspensions were spread onto MH agar plates containing ciprofloxacin (1 μg ml^{-1}) (4× the MIC) or streptomycin (3 μg ml^{-1}) (3× the MIC) to assess the numbers of spontaneous mutants that were resistant to ciprofloxacin or streptomycin. Total bacterial counts were also determined by plating onto MH agar plates without antibiotics. The frequency of spontaneous mutation was calculated by dividing the bacterial count on MH agar plates containing antibiotics by the total bacterial count.

Development of FQ-resistant (FQ^r) mutants under in vitro antibiotic treatment. Cultures of wild-type NCTC 11168 and JB201 grown overnight were resuspended in MH broth to an OD_{600} of 0.05. The bacterial suspensions were cultured microaerobically at 37°C with shaking (200 rpm). Afer 8 h of incubation, ciprofloxacin was added to the media at a final concentration of 1 μg ml^{-1}, and the cultures were continuously incubated for another 40 h. At 0, 3, 8, 24, and 48 h after the start of the incubation, bacterial samples (100 μl each) were taken and spread onto MH agar plates containing ciprofloxacin (1 μg ml^{-1}) to enumerate the number of FQ^r mutant. The total bacterial number was also counted by serial dilutions of the samples and plating onto MH agar plates without antibiotics.

Development of FQ^r mutants under in vivo antibiotic treatment. Five-day-old chickens were divided into two groups, and each group consisted of 10 chickens. One group was inoculated with wild-type NCTC 11168, and the other was inoculated with JB201. Each chicken received approximately 1×10^6 CFU of *C. jejuni*, which were freshly grown in MH broth overnight. Before inoculation, the absence of *Campylobacter* in the chickens was confirmed by culturing cloacal swabs on MH agar plates containing *Campylobacter*-selective supplements (SR0232E and SR0117E; Oxoid). Six days after the inoculation, enrofloxacin treatment was initiated, and the antibiotic was given in drinking water (50 mg liter^{-1}) for 5 days. The medicated water was prepared and replaced daily. Fecal

FIG. 2. Sequence alignment of the putative competence domain of Cj1211 with other ComE3 (ComEC) homologs. B. sub, *B. subtilis* ComEC (NCBI accession number BAA12454.1); N. gon, *N. gonorrhoeae* ComA (accession number YP_207438.1); H. pyl, *H. pylori* ComE3 (accession number NP_208153.1); C. jej, *C. jejuni* Cj1211 (accession number NP_282358.1). The competence domain is based on a report by Draskovic and Dubnau (13).

samples were collected with cloacal swabs, diluted with MH broth, and plated onto MH agar plates containing *Campylobacter*-selective supplements. In addition, each sample was also plated onto MH agar plates containing ciprofloxacin (4 μg ml^{-1}) to count FQ^r isolates. Representative isolates from each group were randomly selected, used in PCR to confirm the Cj1211 mutation, and tested for MICs of ciprofloxacin using the Etest (AB Biodisk, Solna, Sweden). The differences in the emergence of FQ^r mutants between the wild type and JB201 at each sampling time point were compared with a Student's *t* test.

RESULTS

Identification of Cj1211 as a potential competence protein. According to the genomic sequence of *C. jejuni* NCTC 11168 (43), Cj1211 is predicted to be an integral membrane protein and shares 33% identity and 53% similarity to *H. pylori* ComE3. However, it has low overall homology (less than 15% identity) with other functionally defined ComE3 orthologs such as *B. subtilis* ComEC, *N. gonorrhoeae* ComA, *S. pneumoniae* CelB, and *H. influenzae* Rec-2. Despite the low overall homology with other ComE3 orthologs, the putative competence domain, which was previously described for *B. subtilis* ComEC (13), was relatively conserved (23% identity and 39% similarity with *B. subtilis* ComEC, 19% identity and 36% similarity with *N. gonorrhoeae* ComA, and 38% identity and 58% similarity with *H. pylori* ComE3) (Fig. 2). *C. jejuni* Cj1211 has 419 amino acids, which is similar in size to the ComE3 (417 amino acids) of *H. pylori*. However, other ComE3 homologs are larger, with approximately 700 amino acids. A motif search (http://smart.embl-heidelberg.de/) showed that the C-terminal regions of *B. subtilis* ComEC, *N. gonorrhoeae* ComA, and *H. influenzae* Rec-2 contain a lactamase B domain (7), whereas this domain was absent in *C. jejuni* Cj1211, *H. pylori* ComE3, and *S. pneumoniae* CelB, suggesting possible functional variations among these proteins. Cj1211 was annotated as an integral membrane protein (43). Predictions by multiple programs including PSORTb v.2.0 (http://www.psort.org/psortb/index.html), CELLO v.2.5 (http://cello.life.nctu.edu.tw/), PSLpred (http://www.imtech.res.in/raghava/pslpred/), and Gneg-PLoc (http://202.120.37.186/bioinf/Gneg/) suggested that Cj1211 is a possible inner membrane protein (data not shown). Other *C. jejuni* strains and *Campylobacter* species such as *C. jejuni* RM1221, *C. jejuni* 81116, *C. coli*, *C. upsaliensis*, *C. lari*, *C. curvus*, *C. concisus*, and *C. fetus* possess proteins homologous to Cj1211 (data not shown), suggesting that Cj1211 is highly conserved among campylobacters. Interestingly, the published genomic sequences of *C. jejuni* 81-176 (NCBI accession number NC_008787) and *C. jejuni* doylei 269.97 (accession number NC_009707) did not identify a Cj1211 homolog. However, analysis of the genomic sequences indicated that these two strains possess the DNA sequences encoding proteins homologous to Cj1211 (99% and 97% amino acid identities, respectively). The Cj1211 homologs are located between CJJ81176_1224 and *yihY* in *C. jejuni* 81-176 and between JJD26997_0518 and JJD26997_0519 in *C. jejuni* doylei 269.97. The reason that Cj1211 was not annotated in these two strains was unknown.

The Cj1211 gene appears to be positioned in an operon with three upstream genes (Cj1208, Cj1209, and Cj1210) (Fig. 1A). Cj1208 and Cj1209 overlap by 80 nucleotides, Cj1210 and Cj1211 overlap by 1 nucleotide, and Cj1209 and Cj1210 are separated by 8 nucleotides. Cj1208 and Cj1209 encode proteins of unknown functions, while Cj1210 is a probable membrane protein belonging to the DedA protein family whose function is unknown (43). There is a putative promoter upstream of Cj1208. Based on the consensus RpoD promoter sequence of *C. jejuni* (63), the −10, −16, and −35 regions of the promoter were identified (Fig. 1B). The fact that this promoter was able to express Cj1211 in JB201C (Fig. 1C) indicates that it is functional in *C. jejuni*.

Contribution of Cj1211 to natural transformation. The sequence similarity of Cj1211 to ComE3 suggested that Cj1211 could be a competence protein. To examine this hypothesis, a Cj1211 mutant was constructed and used for natural transformation. The inactivation of Cj1211 did not change the bacterial growth rate in MH broth (data not shown). A mutation of Cj1211 resulted in a significant reduction ($P < 0.01$) in the natural transformation frequency compared with that of the wild type (Table 3). The reduction was at least 2,175-fold and 832-fold when Tet^r DNA and Kan^r DNA were used as the donor DNAs, respectively (Table 3). In fact, no transformants were observed with JB201 under the conditions used in this study. Complementation of JB201 with pJB1211 restored the transformation frequency to the level of the wild type, while the control vector (pRY108) did not have an effect on transformation (Table 3). Together, these results clearly showed that Cj1211 plays an important role in the natural transformation of *C. jejuni*.

Mutation of Cj1211 did not affect DNA binding and uptake. Since the mutagenesis of Cj1211 significantly impaired the natural transformability of *C. jejuni*, an assay of DNA binding and uptake was performed to determine which transformation step was affected by the Cj1211 mutation. The assay was performed with ^{33}P-labeled genomic DNA of *C. jejuni* NCTC 11168. Compared with the wild-type strain, JB201 did not show significant changes in DNA binding and uptake (DNA binding values of 1,470.0 ± 92.6 cpm ml^{-1} for the wild type and 1,699.3 ± 111.6 cpm ml^{-1} for JB201 and DNA uptake values of 213.3 ± 48.1 cpm ml^{-1} for the wild type and 220.7 ± 18.6 cpm ml^{-1} for

TABLE 3. Contribution of Cj1211 to natural transformation in *C. jejuni*

Source of donor DNA	Mean transformation frequency ± SD[a]			
	Wild type	JB201	JB201C	JB201P
BQ304[b]	$(1.74 \pm 0.76) \times 10^{-5}$	$<0.8 \times 10^{-8}$	$(1.41 \pm 0.91) \times 10^{-5}$	$<3.9 \times 10^{-8}$
JB101[c]	$(9.16 \pm 7.34) \times 10^{-6}$	$<1.1 \times 10^{-8}$	NT	NT

[a] Each frequency represents the means ± standard deviations of data from triplicate transformations from a single experiment. In all experiments, differences in transformation frequencies between the wild-type strain and JB201 were consistently observed. NT, not tested due to the presence of the Kan^r gene in the complementing plasmid.
[b] Conferring tetracycline resistance.
[c] Conferring kanamycin resistance.

JB201). This result is in contrast to the finding with ComE3 of *H. pylori*, in which a mutation of CmeE3 caused a significant reduction in both DNA binding and uptake (66), but is similar to observations of *N. gonorrhoeae* and *Pseudomonas stutzeri*, for which mutations of their ComE3 orthologs did not affect DNA binding and uptake (16, 22).

Role of Cj1211 in mediating horizontal transfer of antibiotic resistance determinants. Previous studies have shown that a transfer of antibiotic resistance determinants occurred in bacterial cocultures of *C. jejuni* without supplementing extracellular DNA (10, 62), but the contribution of natural transformation to this process has not been formally demonstrated. Since the Cj1211 mutation abolished natural transformation in *C. jejuni*, we compared the transfer of antibiotic resistance markers in the Cj1211$^+$ background to that in the Cj1211$^-$ background. The coculture of the Cj1211$^+$ strains carrying either *aphA3* (for kanamycin resistance) or *tet*(*O*) (for tetracycline resistance) generated progenies that were resistant to both antibiotics within a few hours (Fig. 3A). PCR analysis of the doubly resistant colonies (10 from each sampling at 3, 8, and 24 h) revealed that all of the tested colonies harbored the *aphA3* and *tet*(*O*) genes inserted into *hipO* and *dcuA*, respectively (data not shown), confirming the horizontal transfer of the antibiotic resistance determinants in the coculture. Washing the bacterial inocula prior to coculture delayed ($P = 0.00047$) but did not abolish the emergence of doubly resistant progenies (Fig. 3B). The addition of DNase I into the coculture to a final concentration of 30 μg ml^{-1} completely prevented the appearance of doubly resistant bacterial cells (Fig. 3C). In contrast to the results obtained with the Cj1211$^+$ strains, the coculture experiment using the Cj1211$^-$ strains (JB203 and JB204) did not result in the appearance of doubly resistant progenies (Fig. 3D). This observation was not due to changes in antimicrobial susceptibility because the Cj1211 mutation did not affect the MICs of kanamycin and tetracycline in JB203 and JB204 (data not shown). Furthermore, the acquisition of *aphA3* or *tet*(*O*) rendered *C. jejuni* highly resistant to kanamycin (MIC > 256 μg ml^{-1}) or tetracycline (MIC = 16 μg ml^{-1}) in both the Cj1211$^+$ and Cj1211$^-$ backgrounds. Together, these results strongly indicate that DNA exchange occurs actively in *Campylobacter* cocultures and that the transfer is mediated by free DNA and natural transformation. These findings also confirmed that Cj1211 plays a critical role in the natural transformation of *C. jejuni* and in the horizontal transfer of genetic materials including antibiotic resistance determinants.

Role of Cj1211 in emergence of FQ^r mutants. *Campylobacter* strains can develop resistance to certain antibiotics through spontaneous mutations in target genes. For instance, the resistance of *Campylobacter* to FQs is mediated by spontaneous mutations in GyrA, which occur at frequencies of 10^{-6} to 10^{-8} (64). However, the detected mutants could include both bona fide spontaneous mutants and transformants, because cell death naturally occurs in bacterial cultures, and the DNA released from dead FQ^r cells is conceivably available for the transformation of the wild-type cells. To determine if natural transformation influences the frequency of emergence of FQ^r mutants in *Campylobacter*, we compared the rates of mutant

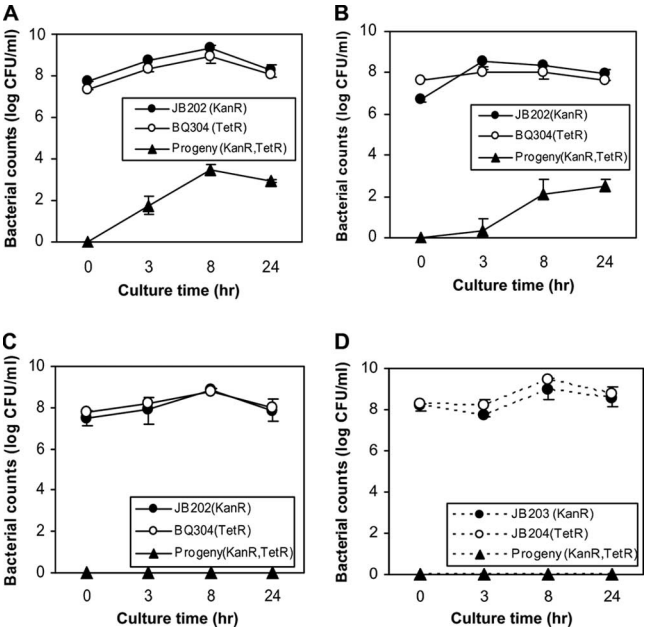

FIG. 3. Natural transformation-mediated transfer of antibiotic resistance determinants in *Campylobacter* cocultures. (A) Coculture of *C. jejuni* JB202 (Kan^r) and BQ304 (Tet^r) in MH broth with no antibiotics. The numbers of bacterial cells that were resistant to both kanamycin and tetracycline were determined on plates containing both antibiotics. (B) Effect of washing the inocula on the development of doubly resistant progenies. JB202 and BQ304 were washed with MH broth five times before being inoculated into the coculture. (C) Effect of DNase I treatment on the development of doubly resistant progenies. DNase I was added to the coculture (at a final concentration of 30 μg ml^{-1}) of JB202 and BQ304. (D) Coculture of *C. jejuni* JB203 (Kan^r) and JB204 (Tet^r) in the absence of DNase I treatment. The Cj1211 gene in both strains was inactivated by insertional mutagenesis. See Table 1 for detailed information on the strains used in this experiment. The results show the means ± standard deviations of data from triplicate samples in a single experiment. Similar patterns of differences were observed in three independent experiments.

TABLE 4. Effect of Cj1211 mutation and DNase I treatment on the emergence of spontaneous mutants that are resistant to FQ and streptomycin

Type of spontaneous mutant	Mean spontaneous mutation frequency ± SD[a]	
	Wild type	JB201
FQ^r mutants[b]	$(3.0 \pm 1.5) \times 10^{-7}$	$(9.8 \pm 5.2) \times 10^{-9}$
FQ^r mutants with DNase I treatment[c]	$(1.9 \pm 1.1) \times 10^{-7}$	NT
Str^r mutants[d]	$(4.4 \pm 3.0) \times 10^{-6}$	$(3.4 \pm 2.2) \times 10^{-8}$

[a] Means ± standard deviations of data for triplicate samples of a single experiment. NT, not tested; Str, streptomycin.
[b] The concentration of ciprofloxacin used is 1 μg ml^{-1}; the experiment was repeated five times.
[c] DNase I was added to the bacterial suspension at a concentration of 100 μg ml^{-1}; the experiment was repeated six times.
[d] The concentration of streptomycin for plating was 3 μg ml^{-1}; the experiment was repeated five times.

emergence in the wild type and JB201. Interestingly, JB201 exhibited a significant reduction (approximately 31-fold) in the frequency of emergence of mutants resistant to ciprofloxacin (Table 4). This reduction in mutation frequency was not due to the change in the susceptibility to ciprofloxacin because the FQ^r mutants from JB201 and the wild type had the same MICs, and the inactivation of Cj1211 in FQ^r mutants did not reduce the MIC of ciprofloxacin (data not shown). In order to confirm whether the reduced emergence of FQ^r mutants in JB201 was linked to natural transformation, the bacterial culture of the wild-type strain was treated with DNase I to remove free DNA. DNase I treatment has been commonly used to assess the function of natural transformation (42, 50, 51). DNase I was added to the bacterial culture to a final concentration of 100 μg ml^{-1}, which was 3.3 times higher than the concentration used to inhibit the transfer of Tet^r and Kan^r by natural transformation (Fig. 3D). The DNase I treatment did not result in a significant reduction in the frequency of FQ^r mutants (Table 4). This result suggests that free DNA was not a major player in the emergence of FQ^r mutants, and the reduced emergence of FQ^r mutants in JB201 could not be explained by its defect in natural transformation. In addition, we examined the development of spontaneous mutants that are resistant to streptomycin, to which the resistance is associated with point mutations in the *rpsL* gene (34). JB201 showed approximately a 129-fold decrease in the frequency of emergence of streptomycin-resistant mutants (Table 4), suggesting that the Cj1211 mutation decreases the general spontaneous mutation rates in *C. jejuni*.

Effect of the Cj1211 mutation on development of FQ^r *Campylobacter* mutants under in vitro treatment. Previously, it was shown that FQ^r *Campylobacter* strains rapidly developed from an FQ-susceptible population when treated with FQ antimicrobials (36, 37, 45). The selection of preexisting FQ^r mutants by the treatment is certainly involved in the development of resistance, but the role of natural transformation in resistance development is unknown. Since Cj1211 contributes to natural transformation and the emergence of spontaneous FQ^r mutants, we investigated if mutations of Cj1211 affected the development of FQ^r mutants when treated with ciprofloxacin. As shown in Fig. 4, the number of spontaneous FQ^r mutants was much lower in JB201 than that in the wild-type strain prior to exposure to ciprofloxacin. After ciprofloxacin treatment was initiated, the FQ^r populations from the wild type and JB201 were both increased. Although the number of FQ^r mutants from JB201 was lower than that from the wild-type strain during the first hours of treatment, the mutant numbers for both strains were similar after 40 h of antibiotic treatment. This result suggests that the development of FQ^r mutants under FQ treatment is dominated by selection and enrichment, and natural transformation does not affect the result of selection.

Effect of the Cj1211 mutation on the emergence of FQ^r mutants under antibiotic treatment in vivo. To determine the effect of the Cj1211 mutation on the emergence of FQ^r mutants under in vivo conditions, two groups of chickens were inoculated with the wild type or JB201 and treated with a therapeutic dose of enrofloxacin (50 mg $liter^{-1}$ in drinking water). JB201 colonized chickens as efficiently as the wild type (Fig. 5A). Enrofloxacin treatment greatly decreased the colonization levels of both the wild type and JB201 but did not eliminate the two strains from the chickens (Fig. 5A). In fact, the *Campylobacter* populations rebounded after the initial decline. Prior to the treatment, no FQ^r mutants were detected in either group. One day after the initiation of antibiotic treatment, FQ^r mutants could be detected in a few of the treated chickens (Fig. 5B). The numbers of FQ^r mutants and chickens carrying FQ^r *Campylobacter* steadily increased during the treatment. At the last sampling time, all *Campylobacter* strains in the chickens were FQ^r (Fig. 5A and B). There were no significant differences ($P > 0.05$) between the two groups in the levels of total *Campylobacter* and FQ^r mutants. This finding indicated that the Cj1211 mutation did not affect the development of FQ^r mutants in chickens treated with enrofloxacin, which was consistent with the result from the in vitro study (Fig. 4). Representative *Campylobacter* colonies selected from each group were examined for ciprofloxacin MIC by Etest. Except for the isolates collected on day 7 (1 day after initiation

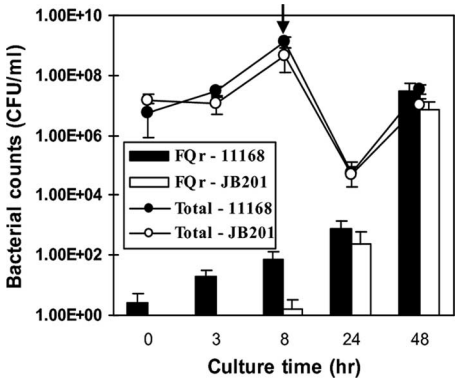

FIG. 4. Effect of the Cj1211 mutation on the development of FQ^r mutants in cultures treated with ciprofloxacin. *C. jejuni* NCTC 11168 and JB201 were grown in separate culture tubes without ciprofloxacin for 8 h, and ciprofloxacin was then added to the cultures to a final concentration of 1 μg ml^{-1}. The initiation time of ciprofloxacin treatment is indicated by an arrow. Open and solid circles indicate the total bacterial counts of JB201 and NCTC 11168, respectively, while the open and solid bars represented the numbers of FQ^r mutants of NCTC 11168 and JB201, respectively. The results show the means ± standard deviations of data for triplicate samples in a single experiment. Similar patterns of results were obtained in three independent experiments.

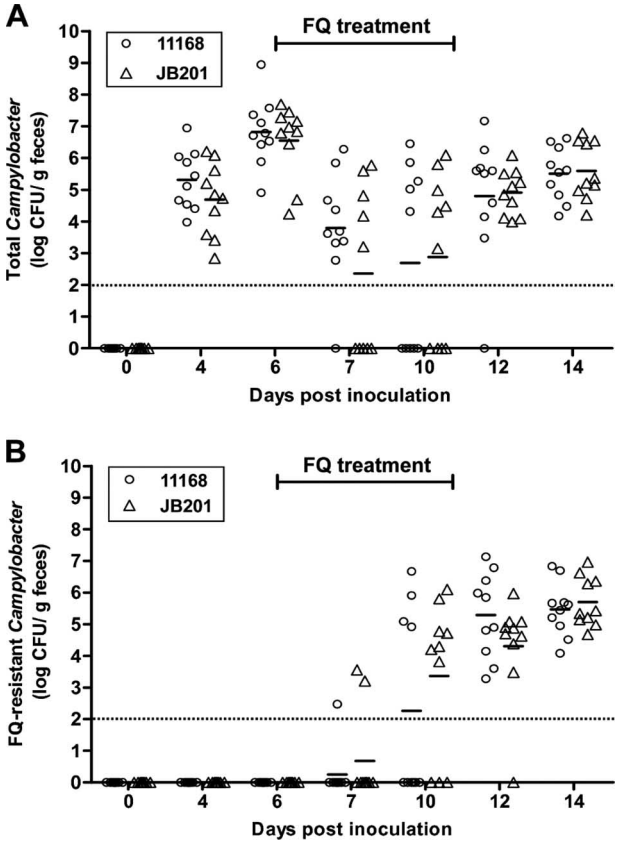

FIG. 5. Effect of the Cj1211 mutation on the development of FQ^r mutants in chickens treated with enrofloxacin. Two groups of chickens (10 birds/group) were inoculated with wild-type NCTC 11168 (circles) and JB201 (triangles), respectively. (A) Total *Campylobacter* numbers detected in the chickens. (B) Numbers of FQ^r *Campylobacter* CFU detected in the chickens. The period of FQ treatment (from 6 to 11 days postinfection) is indicated with a bar above each panel. Each symbol represents the *Campylobacter* number in a single bird. Some symbols are superimposed. The mean of each group is indicated by a horizontal bar. The detection limit is approximately 100 CFU/g feces and is indicated by a dotted line.

of treatment), the majority of the tested isolates from the later sampling times had ciprofloxacin MICs of >32 μg ml^{-1}, and a few had ciprofloxacin MICs of 4 μg ml^{-1}. In addition, some isolates from the JB201-inoculated group were tested by PCR, and all of the tested isolates carried the insertional mutation in Cj1211, indicating that the mutation was stably maintained during in vivo colonization.

DISCUSSION

This study clearly showed that Cj1211 shares significant sequence similarity to ComE3, possesses the competence domain shared by the ComEC (ComE3) homologs (Fig. 2), and is a key player in the natural transformation of *C. jejuni* (Table 3). Based on these findings, we consider Cj1211 to be an inner membrane transporter protein involved in the transfer of DNA across the membrane of *C. jejuni*. Interestingly, the mutation of Cj1211 did not yield measurable differences in DNA binding and uptake (see above). In gram-positive bacteria such as *B. subtilis* and *S. pneumoniae*, the ComEC mutation abolished DNA uptake but did not reduce DNA binding (6, 25). Instead, DNA accumulated on the bacterial surface of the ComEC mutants (6, 49). In gram-negative bacteria, the mutagenesis of ComEC (ComE3) homologs resulted in conflicting results about DNA binding and uptake. Although the *H. pylori* ComE3 mutant exhibited a significant reduction in both DNA binding and uptake (66), the mutation of ComE3 orthologs in *N. gonorrhoeae* and *P. stutzeri* significantly impaired the natural transformation of these bacteria without affecting DNA binding and uptake (16, 22). The discrepancy in DNA uptake between gram-negative and gram-positive bacteria may be explained by the differences in the membrane structures and transformation processes between these two groups of bacteria. In gram-positive bacteria, the mutation of ComEC would directly impair DNA uptake by blocking DNA transfer from the external space to the bacterial cytoplasm. However, in gram-negative bacteria, ComE3 homologs are inner membrane proteins, and the mutation of these proteins should still allow DNA to be transferred to the periplasmic space. Despite the inability of ComE3 mutants to transfer the transforming DNA from the periplasm to the cytosol, the total amount of DNA within bacterial cells may still be comparable to that in wild-type strains. This possibility is likely, because the DNA uptake assay measures the total radioactivity within bacterial cells without differentiating the DNA in the periplasmic space from the DNA in the cytosol. Thus, the commonly used DNA uptake method may not be sensitive enough to determine the role of ComE3 homologs in DNA internalization into the cytosol of gram-negative bacteria.

In our previous study, it was shown that *C. jejuni* possesses a ComEA homolog (Cj0011c), which is a periplasmic DNA receptor binding to both double-stranded and single-stranded DNA with a greater affinity for double-stranded DNA than for single-stranded DNA (29). The mutation of Cj0011c resulted in about a 10-fold reduction in the transformation frequency (29). An early study showed that the mutation of *recA* completely abolished the transformation of this bacterium, indicating that *recA* plays a critical role in the natural transformation of *Campylobacter*, probably by influencing the recombination step (23). Mutation of VirB10, an N-glycosylated protein encoded by the pVir plasmid, decreased the transformation frequency in *C. jejuni* 81-176 (4, 35). In addition, mutation of the N-linked protein glycosylation pathway resulted in a significant reduction (10,000-fold) in transformation frequency in *C. jejuni* (35). Recently, transposon mutagenesis identified a dozen of genes involved in natural transformation (61), several of which are involved in DNA uptake. Findings in our study identified a new competence protein that likely forms an inner membrane channel for the passage of transforming DNA into the cytosol.

Horizontal gene transfer contributes to the intercellular exchange of genetic information and is mediated by natural transformation, conjugation, and transduction (52, 55). Natural transformation has long been recognized in *Campylobacter* and is regarded as being a main mechanism for mediating genetic exchange and diversity in *Campylobacter* (10, 32, 59, 61, 62). Previously, it was shown that the exchange of chromosomally encoded antibiotic resistance determinants occurred in *Campylobacter* cocultures and in chicken gut colonized by *Campylobacter* (10, 62). Although natural transformation was

suspected to be a major player in genetic exchange, its role in mediating the horizontal transfer of antibiotic resistance was not formally demonstrated. In this study, we showed the key role of natural transformation in mediating DNA transfer occurring in *Campylobacter* cocultures (Fig. 3). This conclusion is based on the fact that the addition of DNase I to the coculture and the mutation of Cj1211 abolished the development of doubly resistant mutants (Fig. 3C and 3D). The free DNA available for natural transformation in the cocultures was likely released from *Campylobacter* cells. However, it was unknown if the free DNA was from dead cells or was secreted by growing cells. Since *Campylobacter* is widely present in animals and birds, and mixed infection by multiple *Campylobacter* strains can occur in animal hosts, it is conceivable that natural transformation directly contributes to the spread of antibiotic resistance among different *Campylobacter* strains.

FQ antimicrobials are important for the clinical treatment of campylobacteriosis. However, *Campylobacter* has become increasingly resistant to FQ antimicrobials. In *Campylobacter* strains, resistance to FQs is conferred by gyrase mutations and the function of the multidrug efflux pump CmeABC (19, 36, 57). *Campylobacter* does not have ParC and ParE, and single point mutations in the *gyrA* gene can confer high-level resistance to FQ antimicrobials (36, 44, 45, 47, 57). FQ resistance occurs at a rate as high as 10^{-6} from an FQ-susceptible population (64), but it is technically difficult to directly know if the detected mutants are spontaneous mutants, transformation-generated mutants, or both. To address this question, we added DNase I to the bacterial cultures, and this treatment did not result in a significant change in the frequency of emergence of FQ^r mutants (Table 4), indicating that natural transformation does not play a major role in the emergence of FQ^r mutants. Interestingly, the insertional mutagenesis of Cj1211 substantially reduced the frequency of emergence of FQ^r mutants (Table 4), but the reduction cannot be explained by the defect of the Cj1211 mutant in natural transformation because DNase treatment did not influence the rate of mutant emergence. However, it is possible that Cj1211, a predicted inner membrane protein, may interact with other cytoplasmic proteins involved in DNA transformation and recombination and thus affect both spontaneous mutation and transformation in *Campylobacter*. In *B. subtilis*, competence proteins (ComGA, ComFA, YwpH, and RecA) colocalize at the bacterial poles (26, 31), suggesting spatial proximity or protein interactions between membrane-associated competence proteins and cytoplasmic proteins (e.g., RecA). A recent study indeed indicated that membrane-associated competence proteins (ComEC, ComFA, ComGA, and ComEA) interact with cytoplasmic soluble proteins (RecA, DprA, SsbB, and YjbF) and that a deletion of one competence protein destabilizes the partner proteins and the functional complex in *B. subtilis* (33). If Cj1211 forms a functional complex with other cytoplasmic proteins in *C. jejuni*, such as RecA, which is involved in both natural transformation and spontaneous mutation (5, 8, 54), the loss of Cj1211 may affect the function of RecA and consequently influence the spontaneous mutation rates. This possibility is high because the mutagenesis of Cj1211 also reduced the emergence of streptomycin-resistant mutants (Table 4), suggesting that the general mutation rate was decreased in the Cj1211 mutant. How Cj1211 interacts with other competence proteins in *C. jejuni* will be examined in future studies.

Multiple studies showed that when *Campylobacter*-infected animals were treated with FQ antimicrobials, FQ^r *Campylobacter* mutants rapidly emerged and populated the treated animals (11, 28, 36, 37). The rapid emergence of FQ^r mutants under selective pressure may have contributed to the high prevalence of FQ^r *Campylobacter* strains on a worldwide scale. The development of FQ^r *Campylobacter* from an FQ-susceptible population under FQ treatment certainly involves the selection of preexisting spontaneous mutants, but whether natural transformation is involved in the process is unknown. By taking advantage of the Cj1211 mutant, which is defective in natural transformation, we conducted both in vitro experiments and animal studies to determine if natural transformation affects the development of FQ^r mutants under selection pressure. Prior to exposure to ciprofloxacin, the number of naturally occurring FQ^r mutants in the bacterial population was quite low (Fig. 4). Once the antibiotic treatment was initiated, the majority of the FQ-susceptible bacterial cells were killed, and a large amount of free DNA would be released from the dead cells into the culture supernatant. It is conceivable that the free DNA would be available to transform the FQ^r cells, but the transformants would be quickly killed by ciprofloxacin. Thus, the continuous presence of ciprofloxacin in the cultures eventually eliminated the impact of natural transformation and enriched the FQ^r population (Fig. 4). The in vitro findings were confirmed by the animal treatment study, which showed that the dynamics of development of FQ^r *Campylobacter* were similar between the wild-type strain and JB201 when the chickens were treated with enrofloxacin (Fig. 5). Together, these results suggest that natural transformation does not have a major impact on the development of FQ^r *Campylobacter* strains under FQ-selective pressure.

In conclusion, our study identified Cj1211 as being a new competence factor in *C. jejuni* and proved the role of natural transformation in the horizontal transfer of antibiotic resistance determinants. In addition, we showed that Cj1211 influences the emergence of spontaneous FQ^r mutants, but this effect appears to be independent of its role in natural transformation. Furthermore, we showed that natural transformation does not play a significant role in the development of FQ^r *Campylobacter* strains resulting from FQ treatments. These findings provide new insights into the mechanisms of natural transformation and antibiotic resistance development in *Campylobacter*.

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