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Role of Efflux Pumps and Topoisomerase Mutations in Fluoroquinolone Resistance in *Campylobacter jejuni* and *Campylobacter coli*

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Point mutations in the topoisomerase (DNA gyrase A) gene are known to be associated with fluoroquinolone resistance in *Campylobacter*. Recent studies have shown that an efflux pump encoded by *cmeABC* is also involved in decreased susceptibilities to fluoroquinolones, as well as other antimicrobials. Genome analysis suggests that *Campylobacter jejuni* contains at least nine other putative efflux pumps. Using insertional inactivation and site-directed mutagenesis, we investigated the potential contributions of these pumps to susceptibilities to chloramphenicol, ciprofloxacin, erythromycin, and tetracycline in *C. jejuni* and *Campylobacter coli*. Insertional inactivation of *cmeB* resulted in 4- to 256-fold decreases in the MICs of chloramphenicol, ciprofloxacin, erythromycin, and tetracycline, with erythromycin being the most significantly affected. In contrast, inactivation of all other putative efflux pumps had no effect on susceptibility to any of the four antimicrobials tested. Mutation of *gyrA* at codon 86 (Thr-Ile) caused 128- and 64-fold increases in the MICs of ciprofloxacin and nalidixic acid, respectively. The replacement of the mutated *gyrA* with a wild-type *gyrA* allele resulted in a 32-fold decrease in the ciprofloxacin MIC and no change in the nalidixic acid MIC. Our findings indicate that CmeABC is the only efflux pump among those tested that influences antimicrobial resistance in *Campylobacter* and that a point mutation (Thr-86-Ile) in *gyrA* directly causes fluoroquinolone resistance in *Campylobacter*. These two mechanisms work synergistically in acquiring and maintaining fluoroquinolone resistance in *Campylobacter* species.

Campylobacter is a leading cause of food-borne bacterial infections throughout the world (7). Although most infections are self-limiting, macrolides and fluoroquinolones are the antimicrobials of choice to treat severe *Campylobacter* infections (39). While macrolide resistance has been reported and remains intermittent, the prevalence of fluoroquinolone-resistant *Campylobacter* has escalated since the late 1980s (6, 8, 26, 37, 40). A study in Spain reported high frequencies of ciprofloxacin resistance (72 to 99%) and erythromycin resistance (34.5 to 81.1%) in their 1997–1998 isolates from animals and humans (37). Among human isolates in Pennsylvania, fluoroquinolone-resistant *C. jejuni*, which was not observed between 1982 and 1992, increased to 40.5% in 2001 (26). Fluoroquinolone resistance in *Campylobacter* is associated with point mutations in the DNA gyrase subunit A gene (*gyrA*) (1, 32, 46). In addition, there is growing evidence that efflux pumps play a role in fluoroquinolone and erythromycin resistance of *Campylobacter* (4, 22). Recently, a multidrug efflux pump, CmeABC, has been identified and characterized in *Campylobacter jejuni* (17, 33). The amino acid sequence of CmeB shows a 41% similarity to that of AcrB (17), a major efflux pump in *Escherichia coli* (20, 41). In wild-type *C. jejuni*, this pump was shown to mediate a 2- to 8-fold increase in the MICs of ampicillin, ciprofloxacin, erythromycin, tetracycline, ethidium bromide, and acridine orange (33) and up to 4,000-fold in the MIC of bile salts (17, 18).

Efflux pumps are major components of the bacterial cell. In *E. coli*, it has been estimated that 15 to 20% of the genome may code for membrane transport proteins (43). At least 300 gene products are proposed to transport known substrates effectively, out of which ~20 to 30 transport antimicrobials and other drugs (35). Five families of multidrug efflux pumps that provide resistance to clinically significant drugs and disinfectants are now known in prokaryotes (30). The ATP-binding cassette superfamily is a very large family that consists of ATP-driven uptake and efflux systems and includes ATP-driven multidrug pumps, such as P-glycoprotein and LmrA from *Lactococcus lactis* (44). The major facilitator (MFS) superfamily is another very large, ancient superfamily that consists of secondary transporters driven by chemiosmotic energy and includes proton/drug antiporters, such as QacA from *Staphylococcus aureus* (36). Both the resistance/nodulation/cell division (RND) and the small multidrug resistance families include proton-driven drug efflux pumps, such as *E. coli* AcrB (20) and EmrE (49), respectively. AcrB functions as a multisubunit complex in association with the outer membrane channel protein TolC and the membrane fusion protein AcrA. The multidrug and toxic compounds efflux (MATE) family consists of sodium ion-driven drug efflux pumps, such as NorM from *Vibrio parahaemolyticus* (25). The CmeABC pump of *C. jejuni* belongs to the RND superfamily. Recent genome-sequencing

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TABLE 1. Putative efflux pumps identified based on the genome sequence of *Campylobacter jejuni* NCTC11168

Gene or gene cluster	Product description	Function	Efflux family	Size(s) (bp)	Nucleotide location in insertional mutant
Cj0035c	Putative efflux protein	Antibiotic resistance	MFS	1,203	297
Cj0309c-Cj0310c	Putative efflux protein	Drug/analogue sensitivity	DMT	315/339	487 on *Cj0310c*
Cj0365c (*cmeC*)-*Cj0366c* (*cmeB*)-*Cj0367c* (*cmeA*)	Putative efflux system	Antibiotic resistance	RND	1,479/3,123/1,104	1525 on *Cj0366c*
Cj0560	Putative integral membrane protein	Multidrug	MATE	1,329	612
Cj0619	Putative integral membrane protein	Multidrug	MATE	1,317	571
Cj1031 (*cmeD*)-*Cj1032* (*cmeE*)-*Cj1033* (*cmeF*)	Putative efflux system	Antibiotic resistance	RND	1,275/741/3,018	159 on *Cj1033*
Cj1173-Cj1174	Putative efflux protein	Drug/analogue sensitivity	DMT	342/309	381 on *Cj1174*
Cj1241	Transport/binding protein	Multidrug	MFS	1,200	537
Cj1257c	Putative efflux pump	Drug/analogue sensitivity	MFS	1,185	556
Cj1687	Putative efflux protein	Antibiotic resistance	MFS	1,272	679

data for *C. jejuni* NCTC11168 indicates the presence of multiple putative drug efflux pumps (29). However, their roles in the antimicrobial resistance of *Campylobacter* have yet to be determined, and a complete microbial genome sequence of *Campylobacter coli* is not yet available. The objectives of this study were to identify these putative drug efflux pumps in *C. jejuni* and *C. coli* and to determine their roles, as well as that of a site-directed DNA *gyrA* point mutation, in fluoroquinolone resistance in *Campylobacter*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Two versions of *Campylobacter jejuni* human clinical isolate 81-176 (3) were kindly provided by Qijing Zhang and Patricia Guerry and were designated 81-176 and 81-176G, respectively. Strains 81-176ery, 81-176cip, and 81-176Gcip were derived from 81-176 or 81-176G as the parent strain by in vitro spontaneous mutation selection on Mueller-Hinton agar (BD Diagnostic Systems, Sparks, MD) plates containing erythromycin (ERY) or ciprofloxacin (CIP) ranging from 2 to 16 times the MIC of the parent strains. *Campylobacter coli* strains 124 and 241, resistant to both ERY and CIP, were isolated from turkey and chicken meats, respectively (50).

Campylobacter jejuni and *C. coli* strains were routinely cultured on Mueller-Hinton agar or blood agar plates at 37°C or 42°C under microaerophilic conditions (85% N_2, 10% CO_2, and 5% O_2). *Escherichia coli* DH5α as a host strain for plasmid vectors was grown aerobically in Luria-Bertani medium (Sigma-Aldrich, Co., St. Louis, MO) at 37°C. To select for recombinants, the growth medium was supplemented with either ampicillin (100 μg/ml), chloramphenicol (CHL; 20 μg/ml), or kanamycin (30 μg/ml) as needed.

Identification of putative efflux pumps. Putative efflux pumps of *Campylobacter* (Table 1) were identified using the published genomic sequence database of *C. jejuni* NCTC11168 (http://www.sanger.ac.uk/Projects/C_jejuni/Cj_gene_list_hierarchical.shtml; 29). Coding regions suggestive of membrane transport proteins were determined using TransportDB (http://www.membranetransport.org; 35) and the operon predictions database (http://www.tigr.org/tigr-scripts/operons/pairs.cgi?taxon_id=110).

PCR and reverse transcription (RT)-PCR of putative efflux genes were used to confirm the presence and expression of the putative efflux genes identified. Chromosomal DNA was isolated using a chromosomal DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA). Total RNA was extracted with an RNeasy kit (QIAGEN, Valencia, CA). RT-PCR was conducted with the Access RT-PCR system (Promega, Madison, WI) following the recommendations of the manufacturer. The same sets of primers were used for both PCR and RT-PCR, except for *Cj0309c-Cj0310c* and *Cj1173-Cj1174*, where primers encompassing the entire gene cluster were used for PCR and individual gene primers were used for RT-PCR (Table 2). Each PCR mixture contained 1× PCR buffer, 0.2 mM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase, 1 μM of each primer, and 5 μl of DNA template in a total reaction volume of 50 μl. PCR was conducted using 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension of 72°C for 7 min in a GeneAmp PCR System 9600 (Perkin-Elmer, Foster City, CA).

Construction of gene deletions of putative efflux pumps. Ten putative efflux pump genes or gene operons (Table 1), including *cmeABC*, were selected as targets for insertional mutagenesis. Individual mutants were generated by disrupting the coding regions with a *Campylobacter* chloramphenicol resistance (Cm^r) or kanamycin resistance (Kan^r) cassette. Resistance cassettes were obtained by excision from their host plasmids, pRY109 (48) and pILL600 (16), using PvuII and SmaI digestion, respectively. Cm^r was used for mutation of all 10 genes, whereas Kan^r was also used for the mutation of *cmeB* and *Cj1033* in order to measure possible changes in chloramphenicol susceptibility.

The restriction and modifying enzymes used were purchased from New England Biolabs (Beverly, MA). Target gene sequences were amplified by PCR using *C. jejuni* 81-176 as a template and primers listed in Table 2 and cloned into the plasmid vector pCR2.1 TOPO (Invitrogen, Carlsbad, CA) or pT7Blue (Novagen, San Diego, CA). Cloned target sequences were linearized with a restriction enzyme (AflII, BsmFI, ClaI, EcoRV, NcoI, NdeI, SspI, or SwaI, depending on the target gene), blunt ended by Klenow polymerase where necessary, and ligated with either Cm^r or Kan^r. Recombinants were introduced into *E. coli* DH5α by electroporation. The orientation of the resistance marker was confirmed by PCR to be the same as that of the target efflux gene, which was previously shown to be nonpolar (45). The recombinant plasmid was harvested from *E. coli* DH5α and used to transform *C. jejuni* 81-176 via both natural transformation and electroporation following published protocols (11, 47). To construct mutants of additional *C. jejuni* or *C. coli* strains, the chromosomal DNAs of 81-176 mutants were purified and used to transform these strains using a standard biphasic natural transformation method (47). Successful transformation and recombination were confirmed by PCR amplification using target gene primers flanking the inserted cassette, which showed larger amplification bands (0.8 and 1.4 kb greater for Cm^r and Kan^r insertions, respectively) than for the parent strains (data not shown).

Construction of insertional mutants of *Cj1028c* and *gyrA* point mutation/reversion (Thr-86-Ile) in *C. jejuni*. In order to introduce a point mutation in *gyrA* into a fluoroquinolone-susceptible parent strain (*C. jejuni* 81-176 and 81-176G) or reverse a mutated *gyrA* to a wild-type *gyrA* in a fluoroquinolone-resistant parent strain (*C. jejuni* 81-176cip and 81-176Gcip), a Cm^r marker was inserted in the *Cj1028c* gene upstream of *C. jejuni gyrA*. *Cj1028c* encodes a possible purine/pyrimidine phosphoribosyltransferase (29). The Cm^r marker was used to select *gyrA* mutants generated by homologous recombination. An 850-bp fragment containing the 3′ region of *Cj1028c*, the intergenic region, and the *gyrA* sequence

TABLE 2. Primers for generating fragments to be cloned into recombinant plasmids in efflux pump inactivation and *gyrA* mutation studies

Primer name	Sequence (5′-3′)	Product size (bp)	Target gene/cluster
Efflux pump inactivation study[a]			
0035cF	CTGCACTTTCTCATGTAGAA	809	*Cj0035c*
0035cR	GGCATTTACAAGTAAAATCA		
0309cF	**GCGTGGTGATCATGAAAGAA**	**228**	***Cj0309c***
0309cR	**CAGCGATTAAAAAAAGTTTC**		
0309c-10cF	CGCTGTACAAGTAATAATCA	591	*Cj0309c-Cj0310c*
0309c-10cR	TTAGGTGGAATTATAGAGTG		
0366cF	GCAATGATGCAGTTCCAATT	1,166	*Cj0366c* (*cmeB*)
0366cR	GCCTGTGATACTTAATCCAG		
0560F	CTATAGGATCAGCAGTGCAA	944	*Cj0560*
0560R	TGCCTTATTAAAAGCTGTCA		
0619F	GGGATTTGCTTTCTAAGTAT	1,210	*Cj0619*
0619R	CCACGCAAAAACTCATCTGC		
1033F	CGACCTATTACCGTGCTAAT	1,023	*Cj1033*
1033R	GCAAGCAATGAGAGTTGTAC		
1173F	**TTGCTGCTATTGTATTTGAA**	**259**	***Cj1173***
1173R	**CCAACGATAGAAAGAACAAT**		
1173-4F	TTGCTGCTATTGTATTTGAA	584	*Cj1173-Cj1174*
1173-4R	CTGCTATTATCACGATAATG		
1241F	TGGCAGGAGTTTTGGTTGAT	1,050	*Cj1241*
1241R	AAGCAGGGCTTACAAAAAGT		
1257cF	TGGCTTATGCTTTAGGAACT	545	*Cj1257c*
1257cR	CCCTCCAAGTCCTACACCTA		
1687F	CGGATTTAAAGCAGCTGTGT	1,110	*Cj1687*
1687R	ACAAGACCCATTTGTCTAAC		
16S 1a	AATACATGCAAGTCGAACGA	1,004	16s rRNA
16S 1b	TTAACCCAACATCTCACGAC		
gyrA mutation study[b]			
upF	TGCTCTGCTTTTGTGAATTA	850	*Cj1028c* and *gyrA* (ACA, Thr-86)
86R	AAACTGCTGTATCTCCATGT		
upF	TGCTCTGCTTTTGTGAATTA	850	*Cj1028c* and *gyrA* (ATA, Ile-86)
86mR	AAACTGCTATATCTCCATGT		

[a] Boldface primers were used for efflux gene RT-PCR only.
[b] Point mutation sites of interest are underlined.

up to the mutation site was amplified by PCR using *C. jejuni* 81-176 as a template and primers listed in Table 2 and cloned into a pT7Blue vector. The modified vector was linearized using EcoRV, which cuts once within the inserted fragment (at nucleotide 320 of *Cj1028c*), ligated with a blunt-ended Cm^r cassette, and electroporated into *E. coli* DH5α. Recombinant plasmids were purified from *E. coli* DH5α and used to transform the parent strains, *C. jejuni* 81-176, 81-176G, 81-176cip, and 81-176Gcip, by electroporation (11). Transformants were screened on agar plates containing chloramphenicol and confirmed by chromosomal DNA amplification of the gene flanking the insertion site. This resulted in either mutation (Thr-Ile; ACA-ATA) or reversion (Ile-Thr; ATA-ACA) of *C. jejuni gyrA* at codon 86 through homologous recombination, which was confirmed by DNA sequencing.

A point mutation at *C. jejuni gyrA* codon 86 was incorporated in each of the reverse PCR primers as shown in Table 2. Construct p86m:cm contained a specific mutation (ACA-ATA) converting Thr to Ile at codon 86 and displayed resistance to CIP and nalidixic acid (NAL). Construct p86:cm contained wild-type *gyrA* and was used to restore quinolone susceptibility in a resistant strain possessing a codon 86 point mutation. P86:cm also served as a control in susceptible strains to demonstrate that neither the presence nor the location of the *Campylobacter* Cm^r cassette in *Cj1028c* had any polar effect on quinolone susceptibility.

Antimicrobial susceptibility testing. Susceptibility testing was performed using agar dilution (9, 23). CHL, ERY, NAL, and tetracycline (TET) were purchased from Sigma-Aldrich; CIP was obtained from Pentex, Miles Inc. (Kankakee, IL). The test range used for each antimicrobial was 0.03 to 512 μg/ml. The resistance breakpoints used were as follows: CHL, ≥32 μg/ml; CIP, ≥4 μg/ml; ERY, ≥8 μg/ml; NAL, ≥32 μg/ml; and TET, ≥16 μg/ml (9). *Campylobacter jejuni* ATCC 33560 was used as the quality control organism.

DNA sequencing. The *gyrA* and 23S rRNA genes of *Campylobacter* parent and mutant strains were amplified using PCRs according to published studies (14, 51, 52) and sequenced at the University of Maryland Center for Biosystems Research. DNA sequences were aligned using the Sequencher program (Gene Codes Corporation, Ann Arbor, MI).

Nucleotide sequence accession numbers. The GenBank accession numbers of the control sequences are L04566, AF092101, and U09611.

RESULTS

Identification of putative efflux pumps. Based on the databases and BlastP searches, we identified 10 putative drug efflux pumps, including the known pump, *cmeABC* (*Cj0365c-Cj0366c-Cj0367c*) (Table 1). These genes or gene clusters encode membrane transport proteins belonging to four families of efflux pumps, drug metabolic transporter (DMT), MATE, MFS, and RND. Of note is *Cj1031-Cj1032-Cj1033*, which belongs to the same RND family as *cmeABC* and has recently been designated *cmeDEF* (34).

Sequence analysis showed similarity of *Cj0035c* (28% similarity) to members of the MFS *bcr*/*cmlA* subfamily, which confer bicyclomycin resistance on *E. coli* (21). Cj0309c, Cj0310c, Cj1173, and Cj1174 were similar (27 to 38% similarity) to an ethidium bromide resistance protein in *Staphylococcus aureus* (38). Cj0560 and Cj0619 were similar (21 to 32% similarity) to multidrug efflux pumps in several bacteria, including *E. coli* and *Bacillus subtilis*. Cj1031/Cj1032/Cj1033 (CmeDEF) was

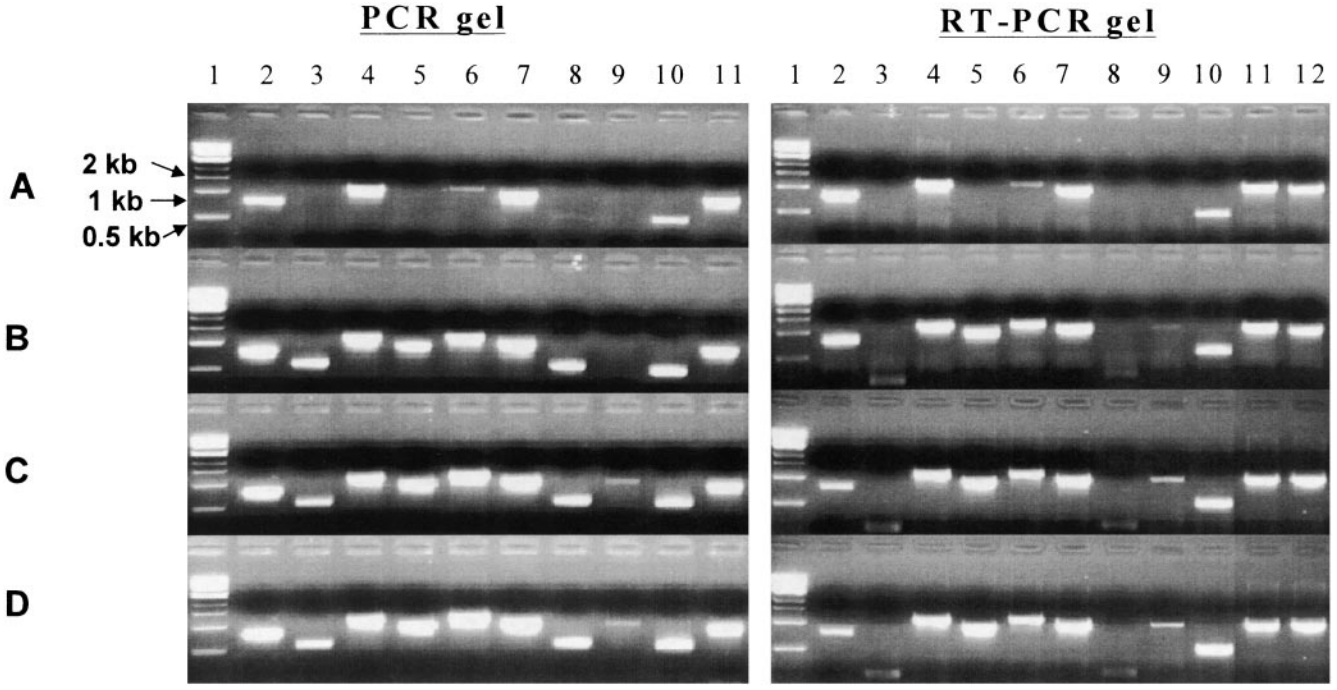

FIG. 1. PCR and RT-PCR amplifications of 10 putative efflux pump genes in four *Campylobacter* strains, *C. coli* 124 (A), *C. coli* 241 (B), *C. jejuni* 81-176 (C), and *C. jejuni* 81-176ery (D). Lanes 1 are 1-kb DNA markers. Lanes 2 to 11 are 10 putative efflux genes: *Cj0035c*, *Cj0309c-Cj0310c*, *Cj0366c*, *Cj0560*, *Cj0619*, *Cj1033*, *Cj1173-Cj1174*, *Cj1241*, *Cj1257c*, and *Cj1687*. In the RT-PCR, *Cj0309c* and *Cj1173* instead of *Cj0309c-Cj0310c* and *Cj1173-Cj1174*, respectively, were amplified, and 16S rRNA (lane 12) served as a control.

36% identical to an efflux system described in *Helicobacter pylori* (HefA/HefB/HefC); however, this putative efflux system was shown not to play an active role in intrinsic antimicrobial resistance in *H. pylori* (2). Several other putative efflux pumps of *Campylobacter* also showed similarity to putative transport proteins in *H. pylori*. Specifically, Cj0560 was similar to HP1184 (26%), Cj1031 to HP0605 (25%), Cj1032 to HP0606 (36%), Cj1033 to HP0607 (37%), and Cj1241 to HP1185 (27%).

In addition, Cj1033 was 26% similar to members of the RND family, such as AcrB/AcrD/AcrF. Cj1241 was similar (26%) to a chloramphenicol resistance protein in *Streptomyces lividans* (5). Cj1257c and Cj1687 were 38% and 23% identical to the multidrug resistance efflux pump PmrA of *Streptococcus pneumoniae* (13) and the NorA quinolone resistance protein of *Staphylococcus aureus* (15), respectively.

Expression of putative efflux pump genes. The presence and expression of the 10 putative efflux pump genes in *C. coli* strains 124 and 241 and *C. jejuni* strains 81-176 and 81-176ery were determined using PCR and RT-PCR. Gene contents and RNA levels varied greatly among the strains tested (Fig. 1). For example, in *C. coli* 124, *Cj0309c-Cj0310c*, *Cj0560*, *Cj1173-Cj1174*, and *Cj1241* were absent from PCR amplification. *Campylobacter jejuni* 81-176 and its derivative 81-176ery possessed all 10 genes; however, not all genes showed bands with similar densities. Interestingly, we did not observe overexpression of any of the 10 putative efflux pumps when comparing antimicrobial-resistant to antimicrobial-susceptible strains.

Effects of gene inactivation on antimicrobial susceptibility. The 10 putative efflux genes/operons were inactivated by inserting *Campylobacter* resistance markers at specific insertion sites (Table 1).

The antimicrobial susceptibilities of the parent and mutant strains are presented in Table 3. All four parent strains were resistant to tetracycline at MICs of ≥32 μg/ml. Strains 81-176ery, 124, and 241 were resistant to both ciprofloxacin and erythromycin at MICs greater than 4 and 16 μg/ml, respectively. When comparing MICs of the parent and mutant strains, the only significant change (>2-fold) in susceptibility to the four antimicrobials tested was observed for *cmeB* mutants, which exhibited a 4- to 256-fold decrease in the MICs of ciprofloxacin, erythromycin, and tetracycline, with erythromycin being the most significantly affected (16- to 256-fold decrease) (Table 3). For mutants with Cm^r inserted, the chloramphenicol MICs were elevated to a similar level (16 to 32 μg/ml). However, in a Δ*cmeB*::Kan^r mutant of *C. coli* 124, the chloramphenicol MIC decreased by eightfold (Table 3). In addition, the *cmeB* mutants reversed from resistant to susceptible phenotypes in *C. coli* 124 against erythromycin and tetracycline, in *C. coli* 241 against erythromycin, in *C. jejuni* 81-176 against tetracycline, and in *C. jejuni* 81-176ery against ciprofloxacin, erythromycin, and tetracycline. Two separate c*meB* mutants of *C. jejuni* 81-176 with either Cm^r or Kan^r inserted showed no difference in susceptibility to ciprofloxacin, erythromycin, or tetracycline (Table 3). There was also no difference in susceptibility to ciprofloxacin, erythromycin, and tetracycline in *Cj1033* mutants with Cm^r or Kan^r insertions, although the insertion orientations of these mutants were in opposite directions.

TABLE 3. Comparison of susceptibilities of *Campylobacter* parent and mutant strains in the putative efflux pump inactivation study

Strain[a]	Method of transformation	MIC (μg/ml)[b]			
		CHL	CIP	ERY	TET
124	NA	16	128	32	512
124-Cj0035c	Natural	64	128	32	512
124-CmeB-Kan	**Natural**	**2 (8)**	**4 (32)**	**0.5 (64)**	**8 (64)**
124-Cj1033	Natural	32	128	32	512
124-Cj1257c	Natural	32	128	32	512
241	NA	8	16	512	256
241-Cj0035c	Natural	32	16	512	256
241-Cj0310c	Natural	32	16	512	256
241-CmeB	**Electroporation**	**32 (NA)**	**4 (4)**	**2 (256)**	**16 (16)**
241-Cj0619	Natural	32	16	512	256
241-Cj1033	Natural	16	16	256	256
241-Cj1174	Electroporation	32	16	512	256
81-176	NA	4	0.125	2	32
Cj0035c	Electroporation	32	0.125	2	32
Cj0310c	Electroporation	32	0.125	2	32
CmeB	**Electroporation**	**16 (NA)**	**0.03 (4)**	**0.125 (16)**	**8 (4)**
CmeB-Kan	**Natural**	**2 (2)**	**0.03 (4)**	**0.125 (16)**	**8 (4)**
Cj0560	Electroporation	32	0.125	2	32
Cj0619	Electroporation	32	0.125	2	32
Cj1033	Electroporation	16	0.125	2	32
Cj1033-Kan	Electroporation	4	0.125	2	32
Cj1174	Electroporation	32	0.125	2	32
Cj1241	Electroporation	16	0.125	2	32
Cj1257c	Electroporation	16	0.125	2	32
Cj1687	Electroporation	16	0.125	2	32
81-176ery	NA	4	4	16	32
Ery-Cj0035c	Electroporation	16	4	16	32
Ery-Cj0310c	Electroporation	16	4	16	32
Ery-CmeB	**Electroporation**	**16 (NA)**	**1 (4)**	**0.125 (128)**	**8 (4)**
Ery-Cj0560	Natural	16	4	16	32
Ery-Cj0619	Electroporation	16	4	16	32
Ery-Cj1033	Electroporation	8	4	16	32
Ery-Cj1174	Electroporation	16	4	16	32
Ery-Cj1241	Electroporation	16	4	16	32
Ery-Cj1257c	Natural	16	4	16	32
Ery-Cj1687	Electroporation	16	4	16	32

[a] Mutations that resulted in >2-fold MIC changes are in boldface.
[b] The numbers in parentheses indicate the differences (*n*-fold) in MICs between parents and their mutant derivatives. The breakpoints used were 32, 4, 8, and 16 μg/ml for CHL, CIP, ERY, and TET, respectively.

DNA sequence analysis of efflux mutants. Identical *gyrA* and 23S rRNA gene sequences were observed in efflux pump mutant and parent strains, indicating that insertional mutation events did not alter these two gene sequences. In *C. coli* 124 and 241 and their respective *cmeB* mutants, a point mutation at amino acid position 86 (Thr-Ile) was identified in the *gyrA* product, whereas the products of *gyrA* of *C. jejuni* 81-176ery and its *cmeB* mutant had a point mutation at amino acid position 90 (Asp-Asn). Both point mutations have been shown to be associated with ciprofloxacin resistance in *Campylobacter* (46). No point mutations in the 23S rRNA genes of *C. coli* 124 and 241 and their respective *cmeB* mutants were identified. Sequence differences at 2172 (T-G) and 2334 (G-A) were detected in the 23S rRNA genes of *C. jejuni* 81-176, 81-176ery, and their respective *cmeB* mutants compared to the sequence in GenBank (accession no. U09611). These nucleotide changes may not be important for erythromycin resistance, since they were observed in both resistant and susceptible *C. jejuni* strains, which may indicate natural sequence polymorphisms existing in the 23S rRNA gene between *C. jejuni* 81-176 and the sequenced strain *C. coli* VC167 (42).

Constructs of site-directed mutagenesis of *C. jejuni gyrA* and the upstream gene *Cj1028c*. Mutagenesis of *Cj1028c* was confirmed by a single 1.65-kb PCR product from the amplification of the gene flanking the insertion site in mutant strains, approximately 800 bp greater than those of the parent strains (data not shown). The replacement of the *gyrA* gene by a point mutation at codon 86 was confirmed by DNA sequencing.

Effect of *gyrA* mutation on quinolone susceptibility in *C. jejuni*. The quinolone susceptibilities of the *C. jejuni gyrA* mutants, along with those of derivative strains restored to the wild-type sequence, were determined by agar dilution. *C. jejuni* mutants (86, G-86, Cip-86m, and Gcip-86m), which incorporated the insertional mutation at *Cj1028c* but remained the same *gyrA* type as their parent strains, had no MIC changes, indicating that the insertion of Cm^r in *Cj1028c* did not affect the susceptibility of the strain to either ciprofloxacin or nalidixic acid (Table 4). However, when *gyrA* Thr-86-Ile mutations were combined with the insertional mutation in *Cj1028c* of *C. jejuni* mutants (86m and G-86m), 128- and 64-fold increases in the MICs of ciprofloxacin and nalidixic acid, respectively, were observed. Interestingly, when the *gyrA* gene was restored to

TABLE 4. MICs of CIP and NAL among *C. jejuni* parent strains 81-176, 81-176G, 81-176cip, and 81-176Gcip and their isogenic mutants replaced with either wild-type *gyrA* (Thr86) or mutated *gyrA* (Ile86)

Strain	Genotype	MIC[a] (μg/ml)	
		CIP	NAL
81-176	Cip^s *gyrA* (Thr-86)	0.125	4
86	Δ*Cj1028c*::Cm^r *gyrA* (Thr-86)	0.125	4
86m	Δ*Cj1028c*::Cm^r *gyrA* (Ile-86)	**16**	**256**
81-176G	Cip^s *gyrA* (Thr-86)	0.5	8
G-86	Δ*Cj1028c*::Cm^r *gyrA* (Thr-86)	0.5	8
G-86m	Δ*Cj1028c*::Cm^r *gyrA* (Ile-86)	**64**	**256**
81-176cip	Cip^r *gyrA* (Ile-86)	128	256
Cip-86m	Δ*Cj1028c*::Cm^r *gyrA* (Ile-86)	128	256
Cip-86	Δ*Cj1028c*::Cm^r *gyrA* (Thr-86)	**4**	256
81-176Gcip	Cip^r *gyrA* (Ile-86)	512	256
Gcip-86m	Δ*Cj1028c*::Cm^r *gyrA* (Ile-86)	512	256
Gcip-86	Δ *Cj1028c*::Cm^r *gyrA* (Thr-86)	**16**	256

[a] >2-fold MIC changes are in boldface.

wild type (Thr-86) in ciprofloxacin-resistant strains (Cip-86 and Gcip-86), the ciprofloxacin MIC decreased 32-fold; however, the nalidixic acid MIC remained unchanged (Table 4).

DISCUSSION

The objectives of this study were to identify efflux pump genes that are associated with antimicrobial resistance in *Campylobacter* and to determine the roles of these efflux pumps and the DNA *gyrA* point mutation in fluoroquinolone resistance in *Campylobacter*. Ten putative efflux pump genes were identified based on bioinformatics data. We were not able to amplify these genes from all *Campylobacter* strains tested, indicating a diverse genetic background within and between *C. jejuni* and *C. coli*. In strains that did amplify these genes by PCR, they did not show PCR bands with the same densities under common culture conditions. Some genes appeared to be constitutively expressed and were common in all the strains tested. These were the genes for Cj0035c, Cj1031/Cj1032/Cj1033, Cj0619, and CmeABC. In addition to the 10 pumps reported in the present study, we examined Cj1296/Cj1297/Cj1298 and Cj1375 but were not successful in generating insertional mutants. Cj1296/Cj1297/Cj1298 is a cluster of hypothetical antimicrobial efflux pumps that is absent in *C. jejuni* 81-176. Cj1375 is a putative member of the MFS family of efflux pumps, which shared similarity with a quinolone resistance NorA protein in *Staphylococcus aureus* (27) and Cj0035c.

We further constructed individual insertional mutations at the 10 identified loci, representing a total of 16 efflux genes. Only mutations in *cmeB* altered susceptibility to the tested antimicrobials, decreasing MICs by 4- to 256-fold. Additionally, *cmeB* inactivation in several resistant strains converted MICs to susceptible levels for ciprofloxacin, erythromycin, and tetracycline. No significant changes in MICs were associated with the other nine putative efflux pumps. These pumps may be responsible for the extrusion of antimicrobials other than the tested or nonantimicrobial substrates. Our findings support the notion that CmeABC constitutes the major multidrug efflux pump system in *Campylobacter*. A closely related gene cluster in the same RND family—*Cj1031*/*Cj1032*/*Cj1033*, which also possesses structural similarity to AcrD in *E. coli*—did not show anticipated decreases in MICs. This agrees with a recent study by Pumbwe et al. showing that Cj1033 (also termed CmeF, a component of the efflux system CmeDEF in *Campylobacter*) does not transport ciprofloxacin (34). In similar studies of *E. coli*, only *acrAB* or *tolC* mutants resulted in significant increases in susceptibilities to a broad range of antimicrobials and compounds tested (28, 41).

To confirm that the mutagenesis procedure did not affect two target genes, *gyrA* and 23S rRNA, in which certain point mutations are associated with ciprofloxacin and erythromycin resistance in *Campylobacter*, DNA sequencing of these genes of parent and mutant strains was conducted, and no change in the DNA sequences was identified. However, although no point mutations were found in 23S rRNA, the erythromycin MICs of *C. coli* 124 and 241 and *C. jejuni* 81-176ery were all above the MIC breakpoint (8 μg/ml), indicating the significant role of efflux pumps in erythromycin resistance in *Campylobater*. This was further confirmed by a greater decrease in erythromycin MICs after the *cmeB* gene was inactivated. Our results are similar to those of Mamelli et al., who reported possible efflux pump involvement in erythromycin resistance based on the finding that an efflux pump inhibitor resulted in significantly increased susceptibilities of the *C. jejuni* reference strain NCTC11168 and several erythromycin-resistant isolates (22).

In *C. jejuni*, point mutation of Thr-86-Ile in *gyrA*, which is homologous to Ser-83-Leu in *E. coli*, was predominantly observed in both clinical and laboratory-derived strains with high-level resistance to ciprofloxacin. Other reported mutations of *gyrA* in *C. jejuni* included Ala-70-Thr (46), Thr-86-Ala (low-level resistance to ciprofloxacin and high-level resistance to nalidixic acid) (1, 24), Thr-86-Lys (19), Asp-90-Asn (1, 12, 31, 32, 46), and Pro-104-Ser (12). Double mutations of *gyrA* combining Thr-86-Ile and Asp-85-Tyr (24), or Asp-90-Asn (12, 31) or Pro-104-Ser (12, 32), have been reported. The role of mutation in *gyrB* has also been examined (1, 31, 32) but is not yet documented in *Campylobacter*. Mutation of Arg-139-Gln in *parC* has been reported in *C. jejuni* by Gibreel et al. (10); however, subsequent studies reported by other investigators failed to confirm that *Campylobacter* possesses a *parC* gene (1, 19, 29, 32). Despite all these observations, direct genetic evidence showing the cause-effect relationship between *gyrA* mutation and fluoroquinolone resistance in *Campylobacter* is lacking.

We adopted an insertional-mutagenesis method and introduced a *Campylobacter* Cm^r cassette and a point mutation of *gyrA* at the chromosomal level to examine the effects of such alterations on the susceptibilities of *C. jejuni* to fluoroquinolones. It appears that the insertion of a Cm^r cassette into the *gyrA* upstream gene *Cj1028c* did not have any detectable effect on the susceptibility of *C. jejuni* strains to either ciprofloxacin or nalidixic acid. Point mutation at codon 86 of *gyrA* significantly increased the MICs of the drugs for *C. jejuni* mutants. In addition, when the wild-type *gyrA* allele replaced the mutated copy in fluoroquinolone-resistant *C. jejuni* strains, the MICs showed significant decreases, although to a lesser extent for ciprofloxacin. This clearly demonstrated a direct causal effect between the Thr-86-Ile point mutation in *gyrA* and fluoroquinolone resistance.

In this study, we also conducted an in vitro spontaneous-mutation selection procedure for the parent strains *C. jejuni* 81-176 and 81-176G. As expected, *C. jejuni* 81-176cip and 81-176Gcip acquired 1,024- and 32- to 64-fold increases in the MICs of ciprofloxacin and nalidixic acid, respectively, and both strains possessed double mutations at codons 86 and 90 in *gyrA*. When point mutation of *gyrA* codon 86 was introduced into the parent strains *C. jejuni* 81-176 and 81-176G, 128- and 32- to 64-fold increases were observed in the MICs of ciprofloxacin and nalidixic acid, respectively. The difference in changes of ciprofloxacin MICs between induction and genetic-manipulation mutants indicated that double mutations at both codons resulted in higher MICs and, more significantly, that other mechanisms, such as overexpression of efflux pumps, may have been involved during the induction procedure. In the study reported by Pumbwe et al., 9 out of 32 multidrug-resistant *C. jejuni* isolates had a mutation at CmeR (Cj0368c, a putative regulator for CmeABC) and overexpressed CmeB and 8 out of 32 were ciprofloxacin resistant but had no mutation in *gyrA* (34).

To our knowledge, this is the first study that has examined the effect of *cmeABC* gene inactivation in *Campylobacter* wild-type strains resistant to clinically important drugs, ciprofloxacin and erythromycin, as well as in *C. coli*. Our findings provided genetic evidence that CmeABC is an important efflux pump in antimicrobial resistance in *Campylobacter*; that a single point mutation, Thr-86-Ile, can render *Campylobacter* resistant to fluoroquinolones; and that these two mechanisms work synergistically in conferring antimicrobial resistance on *Campylobacter*.

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