Evidence for Multiple-Antibiotic Resistance in *Campylobacter jejuni* Not Mediated by CmeB or CmeF

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An efflux system, CmeABC, in *Campylobacter jejuni* was previously described, and a second efflux system, CmeDEF, has now been identified. The substrates of CmeDEF include ampicillin, ethidium bromide, acridine, sodium dodecyl sulfate (SDS), deoxycholate, triclosan, and cetrimide, but not ciprofloxacin or erythromycin. *C. jejuni* NCTC11168 and two efflux pump knockout strains, *cmeB*::Kan^r and *cmeF*::Kan^r, were exposed to 0.5 to 1 μg of ciprofloxacin/ml in agar plates. All mutants arising from NCTC11168 were resistant to ciprofloxacin but not to other agents and contained a mutation resulting in the replacement of threonine 86 with isoleucine in the quinolone resistance-determining region of GyrA. Mutants with two distinct phenotypes were selected from the efflux pump knockout strains. Mutants with the first phenotype were resistant to ciprofloxacin only and had the same substitution within GyrA as the NCTC11168-derived mutants. Irrespective of the parent strain, mutants with the second phenotype were resistant to ciprofloxacin, chloramphenicol, tetracycline, ethidium bromide, acridine orange, and SDS and had no mutation in *gyrA*. These mutants expressed levels of the efflux pump genes *cmeB* and *cmeF* and the major outer membrane protein gene *porA* similar to those expressed by the respective parent strains. No mutations were detected in *cmeF* or *cmeB*. Accumulation assays revealed that the mutants accumulated lower concentrations of drug. These data suggest the involvement of a non-CmeB or -CmeF efflux pump or reduced uptake conferring multiple-antibiotic resistance, which can be selected after exposure to a fluoroquinolone.

Over the past 5 years, *Campylobacter* spp. have been the most common cause of bacterial human gastroenteritis worldwide (1, 2). Infections are usually treated with fluid replenishment. However, in cases of persistent enteritis or extraintestinal infections, a fluoroquinolone or erythromycin is usually the antibiotic of choice (1, 2). In recent years, there has been a worldwide increase in the incidence of both human and veterinary isolates of *Campylobacter* resistant to ciprofloxacin (8, 10, 17, 22, 28). Data indicate that this resistance can be selected for by fluoroquinolones both in vitro and in vivo (26, 27). Fluoroquinolone resistance is usually due to substitutions in the quinolone resistance-determining region (QRDR) of GyrA (28). In *Campylobacter* spp., substitution of threonine 86 for isoleucine in GyrA is most frequently associated with resistance, although other substitutions have also been documented (18, 28). As elsewhere in the world, ciprofloxacin-resistant *Campylobacter* spp. have been isolated in England and Wales (17, 32). Studies in our laboratory have shown that the majority (~90%) of such isolates possess a *gyrA* mutation (28). Between the years 2000 and 2001, ~15% (9,000 cases) of the annual number of *Campylobacter* spp. reported in England and Wales were ciprofloxacin resistant. Our studies estimate that 15% (1,350) of these cases have multiple-antibiotic resistance (MAR) (unpublished data).

Fluoroquinolone resistance can also be conferred by efflux pumps, which limit the access of antibiotics to their targets by actively pumping out these molecules, thereby preventing the intracellular accumulation necessary for lethality (13). Since efflux pumps have broad substrate specificity, they confer MAR (13). MAR bacteria tend to be resistant to at least three classes of structurally unrelated antibiotics. Efflux pumps of the resistance-nodulation-division (RND) family appear to be one of the most widespread antibiotic resistance mechanisms in gram-negative bacteria (4, 24, 29). Efflux was first postulated as a mechanism of multiple-antibiotic resistance in *Campylobacter* spp. 8 years ago (5). In 2002, the efflux system CmeABC in *Campylobacter jejuni* was described for NCTC11168 (30), in which CmeA was the putative membrane fusion protein, CmeB was the putative efflux pump, and CmeC was the putative outer membrane channel protein. This was subsequently confirmed independently by Lin et al. with a poultry isolate (15). CmeB can confer fluoroquinolone resistance and multiple-antibiotic resistance, including antibiotics, detergents, dyes, disinfectants, and bile (15, 30). In other gram-negative bacteria, such as *Escherichia coli*, fluoroquinolone resistance due to efflux can occur synergistically with substitutions in GyrA (25). Recently, Lin et al. (16) demonstrated that CmeB and CmeC are essential for bile tolerance and colonization of chickens by *C. jejuni*.

A second efflux pump, CmeF, linked to a putative outer membrane protein, CmeD, by a putative membrane fusion protein, CmeE, is described in this report.

It has been found for *Staphylococcus aureus* that in the presence of an efflux pump inhibitor the number of norfloxacin-resistant mutants selected in vitro is reduced (19, 20). It was hypothesized that *C. jejuni* lacking an efflux pump(s) would give rise to fewer ciprofloxacin-resistant mutants than a wild-

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TABLE 1. Strains used in this study

Strain no. and group	Description	Source or reference
P270	*C. jejuni* NCTC11168; amenable to genetic manipulation	26
P1031	Ciprofloxacin-resistant mutant selected from P270	This study
P1038	*cmeB*::Kan^r	30
P1035	MAR mutant selected from P1038	This study
P1040	Ciprofloxacin-resistant mutant selected from P1038	This study
P1043	*cmeF*::Kan^r	This study
P1036	MAR mutant selected from P1043	This study
P1045	Ciprofloxacin-resistant mutant selected from P1043	This study
P1048	CmeB overexpressing mutant selected from P270	This study

type strain. It was also hypothesized that mutants derived from a *cmeB*::Kan^r strain would compensate for the loss of CmeB by overexpressing *cmeF*, and vice versa. Therefore, the aims of this study were (i) to characterize a second RND efflux pump, *cmeF*, in *C. jejuni* and (ii) to use ciprofloxacin to select for mutants from NCTC11168, *cmeB*::Kan^r, and *cmeF*::Kan^r strains to determine whether disruption of efflux pump genes affected the frequency of selecting ciprofloxacin-resistant mutants and to characterize any mutants selected.

MATERIALS AND METHODS

Bacterial strains and growth media. *C. jejuni* NCTC11168 (26), *cmeB*::Kan^r (30) and *cmeF*::Kan^r strains, and a mutant of NCTC11168 overexpressing *cmeB* were used throughout this study (Table 1). All strains were routinely cultivated under microaerophilic conditions (5% O_2, 7.5% CO_2, 85% N_2) on Mueller-Hinton (MH) agar plates supplemented with 5% sterile horse blood.

Identification and characterization of CmeF. The efflux pump CmeF was identified and characterized exactly as previously described for CmeB (3, 30). A 1-kb fragment was amplified within the coding region of *cmeF* using primers cmeF-a and cmeF-b (Table 2), and the PCR product was cloned into the pGEM-T Easy vector (Promega). A 100-bp deletion and a unique BglII site were created within the insert by inverse PCR (3, 12, 30) with primers cmeF-c and

TABLE 2. Oligonucleotide primers used in this study

Gene and primer	Nucleotide sequence	Source or reference
16S rRNA gene		
cj16S1	5′-GAGAGGCAGATGGAATTG-3′	This study
cj16S2	5′-CCTACCTCCTATACCTGG-3′	This study
cmeB		This study
cmeB-1	5′-CCTACCTCCTATACCTGG-3′	This study
cmeB-2	5′-TTGAACTTGTGCCGCTGG-3′	This study
cmeF		
cmeF-1	5′-GCACTAATGCACGCATTG-3′	This study
cmeF-2	5′-TGCACCGCTCCTAAGGAA-3′	This study
cmeF-a	5′-TTATCCTAGCCACTCTTG-3′	This study
cmeF-b	5′-CCTATAGAAGCTCCGCCA-3′	This study
cmeF-d	5′-CGC(AGATCT)ATGCGTGCATTA GTGCCT-3′	This study
cmeF-c	5′-CGC(AGATCT)TTAGCGTAGGAA GCATGG-3′	This study
gyrA		
cjgyrA-1	5′-GCCTGACGCAAGAGATGGTT-3′	27
cjgyrA-2	5′-TCAGTATAACGCATCGCAGC-3′	27
gyrB		
cjgyrB-1	5′-ATGGCAGCTAGAGGAAGAG A-3′	27
cjgyrB-2	5′-GTGATCCATCAACATCCGCA-3′	27
porA		
porA-1	5′-TAATGCTGCTGATGGTGG-3′	This study
porA-2	5′-AAGCACCTTCAAGTGTCC-3′	This study

cmeF-d (Table 2). In order to disrupt the gene, a 1.4-kb BamHI restriction fragment containing a kanamycin cassette was then inserted within the BglII site of the *cmeF* insert. The construct was then used to transform the genome sequence of *C. jejuni* strain NCTC11168 and allowed to replace the gene on the chromosome by homologous recombination (3, 12, 30).

tBLAST^N and SWISS-PROT analysis of CmeB and CmeF. Amino acid sequence homology of CmeB and CmeF were determined using the tBLAST^N algorithm. Their putative tertiary structures were compared to the AcrB crystal structure (21) using Swiss-Model software (http://www.expasy.ch).

Selection of ciprofloxacin-resistant and MAR laboratory mutants. Mutants with decreased susceptibility to ciprofloxacin were selected from NCTC11168, *cmeB*::Kan^r, and *cmeF*::Kan^r strains using the method described by Griggs et al. (11). Briefly, the three parent strains were grown overnight in antibiotic-free MH broth, concentrated by centrifugation, and resuspended in sterile MH broth to give a range of inocula (10^5 to 10^9 CFU/ml). Replicate ($n = 5$) agar plates containing ciprofloxacin at two and four times the MICs for the respective parent strains were inoculated with 100 µl of each suspension and incubated for 2 to 5 days under microaerophilic conditions. Ten colonies resembling the morphology of the original strain were randomly chosen from each selecting plate and subcultured onto antibiotic-free media. These were examined for susceptibility and resistance mechanisms.

A *cmeB*-overexpressing mutant was selected by serial passage (increasing the antibiotic concentration with each passage) of NCTC11168 on replicate ($n = 8$) MH agar plates containing cefotaxime at 0.5, 2, and 4 times the MIC. Colonies from the antibiotic-containing plates were emulsified into saline and plated onto fresh antibiotic-free agar plates. The viable-cell count was determined by counting the number of colonies on the antibiotic-free plates. The mutation frequency was determined by dividing the number of colonies on the antibiotic-containing plates by the viable-cell count.

Growth kinetics. Cultures (50 ml) were inoculated with 2 ml of an overnight broth and grown under microaerophilic conditions over a period of 24 h in MH broth, and 1-ml aliquots were withdrawn at 2-h intervals. Cell growth was measured spectrophotometrically by determining the optical density at 600 nm (OD_{600}).

Determination of the MIC. The MICs of the antibiotics ampicillin, chloramphenicol, ciprofloxacin, erythromycin, kanamycin, and tetracycline; the dyes acridine orange and ethidium bromide; the detergents sodium dodecyl sulfate (SDS) and sodium deoxycholate; and the disinfectants cetrimide and triclosan were determined by the agar doubling dilution method as recommended by the NCCLS (23).

Accumulation of ciprofloxacin and ethidium bromide. The concentration of ciprofloxacin accumulated was measured as previously described (30). For ethidium bromide, cells were cultured to mid-log phase (OD_{600}, 0.4 to 0.5), harvested, and washed in sodium phosphate buffer (pH 7.0) to an OD_{600} of 0.2 (10 ml). After the cells were preincubated for 15 min at 37°C, a 2-ml time zero aliquot was withdrawn, and ethidium bromide was added to a final concentration of 2 µg/ml. Further aliquots were withdrawn at 5, 10, 15, and 20 min after the addition of ethidium bromide. The aliquots were stored on ice until the fluorescence was determined. Fluorescence was measured at an excitation of 530 nm and an emission of 600 nm. Accumulation experiments were performed with or without the protonophore carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) added to a final concentration of 150 µM after withdrawal of the 5-min aliquot. All experiments were performed in duplicate on at least three separate occasions. The data were analyzed by Student's *t* test, and a *P* value of ≤0.05 was considered significant.

Sequence analysis of the QRDR of *gyrA* or *gyrB*. Mutation detection by denaturing high-performance liquid chromatography and DNA sequencing were performed as described previously (7). The primers used are listed in Table 2.

Expression of *cmeB*, *cmeF*, and *porA*. Expression of *cmeB*, *cmeF*, and *porA* (which encodes the major outer membrane protein [MOMP]) was measured by comparative reverse transcription-PCR exactly as described previously (6, 31). The primers used are listed in Table 2. *porA* expression was confirmed by SDS-polyacrylamide gel electrophoresis (9).

RESULTS

Identification and characterization of CmeF. A second efflux system, corresponding to regions Cj1031, -1032, and -1033 in the *C. jejuni* NCTC11168 genome sequence, was identified by homology to *E. coli* AcrB, AcrD, and AcrF and *C. jejuni*

TABLE 3. Phenotypes of selected mutants

Strain	Phenotype[a]	MIC (µg/ml)[b]												Accumulation	
		Amp	Cip	Chl	Ery	Tet	EtBr	AO	SDS	Deox	Bile	Cet	Tri	Cip[c]	EtBr[d]
P270	WT	4	0.25	1	0.25	0.25	4	16	8	16	64	8	4	15.9 ± 1	14.6 ± 1
P1031	Cip[r]	2	32	1	0.25	0.25	4	16	8	16	64	8	4	ND[e]	ND
P1048	CmeB[+]	32	8	16	4	8	32	64	64	64	128	64	32	8.3 ± 2	7.4
P1038	CmeB[−]	1	0.06	0.5	0.06	0.06	2	8	2	4	16	2	1	24.4 ± 2	21.7 ± 2
P1040	Cip[r]	1	16	1	0.06	0.06	2	8	2	4	16	2	1	ND	ND
P1035	MAR	1	4	4	0.06	0.06	8	32	8	4	16	2	1	19.8 ± 2	17.98 ± 2
P1043	CmeF[−]	0.5	0.25	1	0.25	0.25	1	4	4	8	32	2	1	17 ± 1	23.2 ± 1
P1045	Cip[r]	0.5	32	2	0.25	0.25	1	4	4	8	32	2	1	ND	ND
P1036	MAR	0.5	4	4	0.25	0.25	8	32	8	8	32	2	1	13.2 ± 2	19.1 ± 1

[a] WT, wild type.

[b] Amp, ampicillin; Cip, ciprofloxacin; Chl, chloramphenicol; Ery, erythromycin; Kan, kanamycin; Tet, tetracycline; EtBr, ethidium bromide; AO, acridine orange; Deox, sodium deoxycholate; Cet, cetrimide; Tri, triclosan.

[c] Micrograms of ciprofloxacin per nanogram of dry cells after 5 min of exposure (± standard deviation).

[d] Fluorescence units of ethidium bromide after 5 min of exposure (± standard deviation).

[e] ND, not determined.

CmeB. The inner membrane efflux pump was designated CmeF (Cj1033), CmeD (Cj1031) the outer membrane protein, and CmeE (Cj1032) the membrane fusion protein. Like CmeB, CmeF was large (3,000 bp) and had a predicted 12-transmembrane (TM) helical domain structure (30). CmeF had a moderate amino acid similarity to the *E. coli* RND efflux pump proteins AcrB, AcrD, and AcrF (26.9, 27.1, and 28.7% identity, respectively). CmeB had higher homology with the *E. coli* proteins (42.2, 43.6, and 42.2%, respectively) (30). CmeF showed only 25% amino acid identity with CmeB. P1043 (*cmeF*::Kan[r]) was more susceptible to ampicillin, ethidium bromide, acridine, SDS, deoxycholate, triclosan, and cetrimide (Table 3). In contrast, P1038 (*cmeB*::Kan[r]) was also more susceptible to ciprofloxacin, erythromycin, and tetracycline (Table 3) (30). Interestingly, and as found for P1038 (*cmeB*::Kan[r]), P1043 (*cmeF*::Kan[r]) was also more susceptible to bile (Table 3). Although the two efflux pumps are RND-type pumps and are predicted to have similar basic tripartite structures, these data suggest that they have different substrate-binding sites and conductance properties.

Protein structure of CmeF. TBLAST[N] comparisons revealed that the amino acid sequence of CmeB was >40% identical to that of AcrB in both the TM and non-TM domain regions (21). However, CmeF was <30% identical to AcrB and CmeB throughout the TM and non-TM regions (data not shown). Swiss-Model analysis also revealed that the CmeB tertiary structure was similar to that of AcrB, whereas the structure of CmeF could not be modeled due to its low structural similarity to AcrB.

Antibiotic susceptibility of ciprofloxacin-resistant mutants and mutations in the QRDR of *gyrA* or *gyrB*. Mutants were selected on agar containing ciprofloxacin from *cmeB*::Kan[r] (P1038) and *cmeF*::Kan[r] (P1043) strains as readily as from NCTC11168 (P270), with similar mutation frequencies of 2.2 × 10^{−9}, 1.7 × 10^{−9}, and 2.5 × 10^{−9}, respectively. Growth curves showed that P1038 (*cmeB*::Kan[r]), P1043 (*cmeF*::Kan[r]), and P1048 and the mutants were able to grow as well as P270 (wild type) under normal laboratory conditions (data not shown).

All mutants (*n* = 8) selected from NCTC11168 (P270) had an ~100-fold decrease in susceptibility to ciprofloxacin. These

mutants also had a mutation resulting in the replacement of threonine at position 86 with isoleucine in GyrA (e.g., P1031). Two mutant phenotypes were selected from each strain (P1038 [*cmeB*::Kan[r]] and P1043 [*cmeF*::Kan[r]]). The first phenotype, illustrated by strains P1040 and P1045, was similar to that of the NCTC11168-derived mutants and had an ~100-fold decrease in susceptibility to ciprofloxacin, a Thr86-Ile GyrA substitution, and no mutations in *gyrB*. The second mutant phenotype, illustrated by P1035 and P1036, had an ~16- to 30-fold decrease in susceptibility to ciprofloxacin and decreased susceptibility to chloramphenicol, ethidium bromide, acridine orange, and SDS. Susceptibilities to erythromycin, tetracycline, deoxycholate, and bile were unchanged. There were no mutations in *gyrA* or *gyrB*. The cefotaxime-selected *cmeB*-overexpressing mutant, P1048, was highly resistant to multiple agents and very resistant to bile (Table 3).

On three separate occasions, disruption of *cmeF* in P1035 and disruption of *cmeB* in P1036 were attempted by using a chloramphenicol resistance cassette (33). No double mutants were obtained. However, disruption of *cmeF* in NCTC11168 with this cassette in parallel gave rise to *cmeF*::Chlor[r] mutants (data not shown). DNA sequencing of *cmeF* in P1035 and *cmeB* in P1036 revealed no mutations in either entire structural gene.

Accumulation of ciprofloxacin and ethidium bromide. Accumulation of ciprofloxacin by P1043 (*cmeF*::Kan[r]) was no different than that of the parent strain (P270) (Table 3). However, accumulation of ethidium bromide increased 1.6-fold compared to that of the wild type (P270) (*P* = 0.0048) (Fig. 1B). CCCP increased the accumulation of ethidium bromide by P1043 by 16% and that of P270 by 48% (data not shown). Compared with the wild-type parent strain (P270), the concentration of ciprofloxacin and ethidium bromide accumulated was also reduced in P1048 (48 and 50%, respectively; *P* = 0.0016 and 0.0017, respectively) (Table 3). CCCP increased the accumulation of ciprofloxacin and ethidium bromide by P1048 to 79.7 and 82.0%, respectively, of that of the wild type (data not shown).

Accumulation assays of the MAR mutants derived from the insertionally inactivated mutants revealed that they accumulated less ciprofloxacin and ethidium bromide than their re-
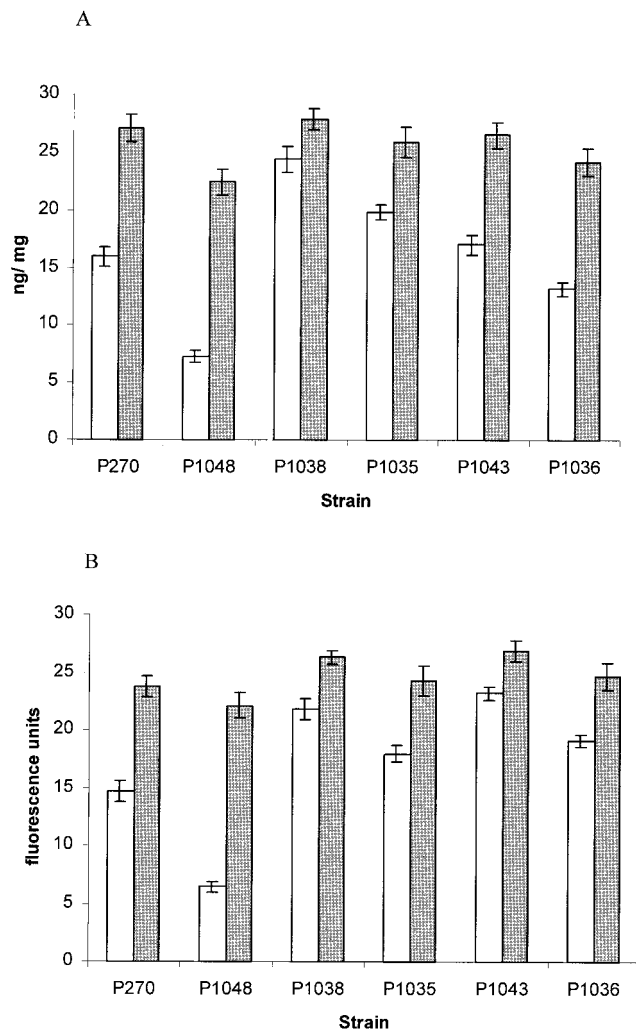
A

B

FIG. 1. Accumulation of ciprofloxacin (A) and ethidium bromide (B) without (open bars) or with (shaded bars) CCCP by P270 (NCTC11168), P1048 (*cmeB*++ [overexpressed]), P1038 (*cmeB*::Kan^r), P1035 (*cmeB*::Kan^r MAR mutant), P1043 (*cmeF*::Kan^r), and P1036 (*cmeF*::Kan^r MAR mutant). The values are from five independent experiments expressed as means ± standard deviations and were obtained at the 15-min time point.

spective parent strains. P1035 accumulated 19% less ciprofloxacin and 11% less ethidium bromide than its parent strain, P1038 ($P = 0.0043$) (Table 3). CCCP increased this concentration in P1035 by 7.1 and 7.9%, respectively (data not shown). However, P1035 still accumulated higher concentrations of ciprofloxacin and ethidium bromide than NCTC11168 (P270) (Table 3). Similarly, P1036 accumulated 23% less ciprofloxacin and 18% less ethidium bromide than P1043 ($P = 0.0049$ and 0.0042). CCCP increased this concentration by 5 and 8%, respectively (Fig. 1). P1036 accumulated 17% less ciprofloxacin than NCTC11168, whereas it accumulated 24% more ethidium bromide.

Expression of *cmeB*, *cmeF*, and *porA*. There was no significant difference between the expression levels of *cmeB* by P1043 and P1036. Similarly, *cmeF* expression by P1038 and P1036 was not significantly different from that of P270 or the ciprofloxa-

cin-resistant mutants (P1038, P1040, and P1045). None of the strains showed any difference in the level of expression of *porA* (data not shown). Only the cefotaxime-selected MAR mutant, P1048, overexpressed *cmeB* (2.4-fold).

DISCUSSION

A second RND-type efflux system, CmeDEF, in *C. jejuni* is described in this study. This system has different substrate-binding properties and is thus functionally distinct from the previously described CmeABC. However, these systems have some overlap in their substrate profiles.

More important is the observation that lack of CmeF also increased sensitivity to bile. This phenomenon was previously observed with two different strains lacking CmeB (15, 30), and recently it has been shown that CmeB is essential for colonization of chickens (16). As no up-regulation of *cmeB* or *cmeF* was detected in the *cmeF*::Kan^r or *cmeB*::Kan^r strain, respectively, it is intriguing that *C. jejuni* should have two proteins involved in bile resistance but that lack of either protein confers susceptibility. These data suggest that the two pumps have low binding specificity for bile.

The hypothesis that ciprofloxacin-resistant mutants would be selected less frequently from the knockout mutants was not supported, as there was no significant difference among the three strains in the frequency of mutation to resistance. In addition, the hypothesis that any selected mutants would compensate for the lack of CmeB by overexpressing *cmeF*, and vice versa, was also not supported. The latter data provide further evidence that *cmeB* and *cmeF* are expressed independently.

Ciprofloxacin selected for mutants with the threonine 86-to-isoleucine substitution in the QRDR of GyrA of NCTC11168 and the *cmeB*::Kan^r and *cmeF*::Kan^r strains. However, ciprofloxacin also selected for MAR mutants derived from P1038 (*cmeB*::Kan^r) and P1043 (*cmeF*::Kan^r), whose substrate profiles were different from that of the cefotaxime-selected MAR *cmeB*-overexpressing mutant (P1048). Compared with P1048, the ciprofloxacin-selected MAR mutants, P1035 and P1036, had more limited substrate profiles. Data from accumulation experiments suggest that these mutants either take up less of these agents or have enhanced efflux. However, this appears not to be mediated by CmeB, CmeF, or PorA/MOMP, the major outer membrane protein (porin) (14). In addition, resistance to bile was not restored in the MAR mutants.

Despite repeated attempts, deletion of *cmeF* in P1035, or of *cmeB* in P1036, was unsuccessful, suggesting that one RND pump is essential for viability. Although double mutants could not be selected, DNA sequencing of *cmeF* and *cmeB* in each mutant revealed no mutations and ruled out substrate specificity alterations or substrate efficiency changes in CmeB or CmeF as a cause of multiple-antibiotic resistance. Ciprofloxacin remains the first- or second-line antibiotic for treatment of *Campylobacter* sp. infections. Therefore, the use of fluoroquinolones may select for resistance not only to these agents, but to other antibiotics. These data have shown that ciprofloxacin can select for resistance to fluoroquinolones. However, this study suggests that fluoroquinolones can also select for MAR mutants. Other work in our laboratory has shown that MAR in *C. jejuni* cannot be fully accounted for by the known mechanisms (31); therefore, it is possible that multiple-antibiotic

resistance may be mediated by another efflux pump. As the *C. jejuni* genome contains at least eight other non-RND efflux pumps that could be involved in the transport of antibiotics, further investigation of these is now warranted.

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