

Critical Role of Multidrug Efflux Pump CmeABC in Bile Resistance and In Vivo Colonization of *Campylobacter jejuni*

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CmeABC functions as a multidrug efflux pump contributing to the resistance of *Campylobacter* to a broad range of antimicrobials. In this study, we examined the role of CmeABC in bile resistance and its contribution to the adaptation of *Campylobacter jejuni* in the intestinal tract of the chicken, a natural host and a major reservoir for *Campylobacter*. Inactivation of *cmeABC* drastically decreased the resistance of *Campylobacter* to various bile salts. Addition of cholate (2 mM) in culture medium impaired the in vitro growth of the *cmeABC* mutants but had no effect on the growth of the wild-type strain. Bile concentration varied in the duodenum, jejunum, and cecum of chicken intestine, and the inhibitory effect of the intestinal extracts on the in vitro growth of *Campylobacter* was well correlated with the total bile concentration in the individual sections of chicken intestine. When inoculated into chickens, the wild-type strain colonized the birds as early as day 2 postinoculation with a density as high as 10^7 CFU/g of feces. In contrast, the *cmeABC* mutants failed to colonize any of the inoculated chickens throughout the study. The minimum infective dose for the *cmeABC* mutant was at least 2.6×10^4 -fold higher than that of the wild-type strain. Complementation of the *cmeABC* mutants with a wild-type *cmeABC* allele in *trans* fully restored the in vitro growth in bile-containing media and the in vivo colonization to the levels of the wild-type strain. Immunoblotting analysis indicated that CmeABC is expressed and immunogenic in chickens experimentally infected with *C. jejuni*. Together, these findings provide compelling evidence that CmeABC, by mediating resistance to bile salts in the intestinal tract, is required for successful colonization of *C. jejuni* in chickens. Inhibition of CmeABC function may not only control antibiotic resistance but also prevent the in vivo colonization of pathogenic *Campylobacter*.

Bile is produced in the liver, stored in the gall bladder, and released into the small intestine for digestion of fats. Bile contains a group of detergent-like bile salts which not only play a role in fat digestion and absorption but also display potent bactericidal activity. Bile salts are amphipathic molecules which can kill bacteria by destroying the lipid bilayer of cell membrane (16, 20, 21). Thus, resistance to bile salts is essential for enteric bacteria to survive in the intestinal tract. To diminish the action of bile salts, enteric organisms (including pathogens and normal intestinal microflora) have evolved multiple mechanisms to resist the bactericidal effect of bile salts. Generally, enteric bacteria can resist bile salts by using efflux pumps, modulating synthesis of lipopolysaccharide and porins, or producing bile salt hydrolase (11, 16). Among these mechanisms, extrusion of bile salts from the bacterial cytoplasm directly out of the cell is considered a main mechanism of bile resistance in gram-negative bacteria and is mediated by multidrug resistance (MDR) efflux systems (16). Several MDR efflux systems in gram-negative bacteria have been demonstrated to confer resistance to bile salts in vitro (9, 12, 17, 32, 48). Despite these advances in understanding the role of MDR pumps in bile resistance, direct evidence that MDR efflux

pumps are required for bacterial adaptation in animal intestines is still lacking. Thus, examining the role of MDR efflux pumps in bacterial survival and colonization in vivo will greatly improve our understanding of the pathophysiological functions of bacterial MDR efflux pumps.

Campylobacter jejuni is the leading bacterial cause of human enteritis in many industrialized countries (13). The majority of human infections result from consumption of undercooked poultry meat or other food products cross-contaminated with raw poultry meat during food preparation (47). As an enteric pathogen, *C. jejuni* enters the host intestine via oral ingestion and colonizes the distal ileum and colon. Once inside the intestine, *C. jejuni* is faced with multiple levels of stresses, such as the action of antimicrobial bile salts and peptides, starvation (e.g., iron limitation), competition with other residential flora, antibiotic treatments, and attack by host immune defenses. *Campylobacter* must counteract these harsh conditions in order to survive and multiply in an animal host. In the past decades, many efforts have been directed to understanding the virulence factors involved in *Campylobacter* adhesion, invasion, and cytotoxicity. Some known examples of putative virulence elements include CDT toxins (18, 19, 26), PEB1 (38), CadF (23), Fla (15, 35), JlpA (22), Cia proteins (24, 43), the pVir plasmid (4), and a phase-variable capsule (5), of which the motility-mediating flagellum (Fla) is the best-characterized virulence factor shown to be required for *Campylobacter* colonization in the gastrointestinal tract of birds and mammals (15, 34, 35, 37, 50). Despite these advances in understanding the pathobiology of *Campylobacter* pathogenesis, little is known about the mechanisms utilized by *Campylobacter* to adapt in the intestinal

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TABLE 1. Bacterial plasmids and strains used in this study

Plasmid or strain	Description	Source or reference
Plasmids		
pGEMT-Easy	PCR cloning vector, Amp ^r	Promega
pCMEC	pGEMT-Easy containing 1.5-kb <i>cmeC</i> fragment, Amp ^r	This study
pCMECK	pCMEC with kanamycin resistance cassette inserted in <i>cmeC</i> gene, Amp ^r Kan ^r	This study
pUOA18	<i>E. coli</i> - <i>C. jejuni</i> shuttle vector, Cm ^r	49
pCME	pUOA18 derivative containing a wild-type <i>cmeABC</i> operon	This study
Strains		
<i>C. jejuni</i>		
81-176	Wild type; isolated from human	7
21190	Wild type; isolated from chicken	25
JL101	21190 derivative; <i>cmeB::kan</i>	28
JL102	21190 derivative; <i>cmeC::kan</i>	This study
JL103	JL101/pCME, Kan ^r Cm ^r	This study
JL104	JL102/pCME, Kan ^r Cm ^r	This study
<i>E. coli</i> JM109	<i>endA1 recA1 gyrA96 thi hsdR17 (r_k⁻ m_k⁺) relA1 supE44 Δ(lac-proAB) [F' traD36 lacI^qΔM15]</i>	Promega

environment in the presence of various antimicrobial agents, such as bile salts. Understanding the adaptation mechanisms may facilitate the development of effective means to prevent and control *Campylobacter* infection in humans and animal reservoirs.

Recently, a *Campylobacter* multidrug efflux pump (named CmeABC) contributing to antimicrobial resistance was characterized (28, 31, 42). This efflux pump is chromosomally encoded by a three-gene operon (*cmeABC*) and shares significant sequence and structural homology with known tripartite multidrug efflux pumps in other gram-negative bacteria. Based on the sequence and structural homology with known bacterial efflux pumps, it was predicted that CmeA, CmeB, and CmeC are a periplasmic protein, an inner membrane drug transporter, and an outer membrane protein, respectively. It is believed that the three members function together and form a membrane channel for the extrusion of antimicrobials and other toxic compounds in *Campylobacter* (28). An insertional mutation in *cmeB* of various *Campylobacter* strains resulted in substantial decreases in *Campylobacter* resistance to various antimicrobials (28, 31, 42). Accumulation assays demonstrated that CmeABC functions as an energy-dependent efflux pump in *C. jejuni*. PCR and immunoblotting showed that *cmeABC* is broadly distributed and constitutively expressed in various *Campylobacter* isolates grown in Mueller-Hinton (MH) broth. Our previous findings (28) also suggested that CmeABC may be an important player in bile resistance, which prompted us to determine its role in the adaptation of *Campylobacter* to the intestinal environment of an animal host. Using both in vitro and in vivo systems, we demonstrated in this study that CmeABC, by mediating bile resistance, is essential for *Campylobacter* growth in bile-containing media and in colonization in animal intestinal tracts. These findings define a key natural function of a multidrug efflux pump in an enteric pathogen and open new avenues for the development of measures to control *Campylobacter* infection in humans and in animal reservoirs.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The various *Campylobacter* strains, mutants, and plasmids used in this study and their sources are

listed in Table 1. These isolates were routinely grown in MH broth (Difco) or agar at 42°C under microaerophilic conditions, which were generated using a *Campypak* Plus (Becton Dickinson) gas pack in an enclosed jar. When needed, culture media were supplemented with kanamycin (30 µg/ml) or chloramphenicol (20 µg/ml).

Insertional mutation of *cmeC*. An isogenic *cmeC* mutant of strain 21190 was constructed by insertional mutagenesis. According to the published complete sequence of the *cmeABC* operon in strain 81-176 (28), primers CF (5'-GGCTTATGAAATTACAGATGCAGA) and CR (5'-TCTTGGGAAAAGAAAACAATAGC) were used to amplify a 1.5-kb fragment that spans from the 5' end to 70 bp downstream of the stop codon of *cmeC*, which contains a unique *Bsr*BRI restriction site in the middle of the open reading frame. The PCR product was cloned into pGEMT-Easy (Promega), resulting in the construct pCMEC. Primers KF (5'-AAAAGATACATATCGATGAATTGTGTCTCA) and KR (5'-AATTGATACATATCCAGTTGGTGATTTTG), which were designed with an attached *Bsr*BRI restriction site (underlined in primers), were used to amplify the 1.1-kb kanamycin resistance cassette from the EZ::TN <KAN-2> Tnp transposon (Epicentre). The PCR product containing the kanamycin resistance cassette was digested by *Bsr*BRI and ligated to the same enzyme-digested pCMEC to obtain the construct pCMECK. Sequencing of the construct indicated that the kanamycin resistance cassette was inserted in the *Bsr*BRI site of *cmeC* and in the same orientation as the *cmeC* gene. The plasmid pCMECK, which served as a suicide vector, was electroporated into *C. jejuni* 81-176. Transformants were selected on MH agar containing 30 µg of kanamycin/ml. Inactivation of the *cmeC* gene in the transformants by insertion of the kanamycin resistance cassette was confirmed by PCR and immunoblotting using anti-CmeC antibodies as described previously (28). To create the isogenic *cmeC* mutant in strain 21190, the insertional mutation in *cmeC* of 81-176 was transferred into strain 21190 by natural transformation as described previously (28). The *cmeC* mutation in 21190 was further confirmed by PCR and immunoblotting (see Fig. 1). Sequence data showed that the kanamycin resistance cassette was inserted within the codon encoding amino acid 154 of CmeC in the same direction as the transcription of *cmeC*. The *cmeC* mutant of 21190 was named JL102 in this study. Generation of the *cmeB* mutant of 21190, named JL101 in this paper (Table 1), was described in a previous study (28).

Complementation of JL101 and JL102. The 6-kb *cmeABC* operon was amplified from strain 81-176 by PCR using primers F and R, which span from the promoter region of *cmeABC* to 70 bp downstream of the stop codon of *cmeC* (28). PCR was performed with *PfuTurbo* DNA polymerase (Stratagene), using a 500 nM concentration of each primer. The annealing temperature used was 50°C. The blunt-ended PCR product was purified using a QIAquick PCR purification kit (Qiagen) and ligated to shuttle vector pUOA18 (49), which was digested with *Sma*I prior to ligation. The ligation mix was introduced into JL101 by electroporation. Transformants were selected on MH agar containing 20 µg of chloramphenicol/ml. One transformant harboring the *cmeABC* operon was identified, and the construct was designated pCME. Sequence analysis indicated that pCME contained intact *cmeB* and *cmeC*. However, the 5'-end 18 bp of *cmeA* were deleted. This deletion did not affect the expression of *cmeB* and *cmeC* from

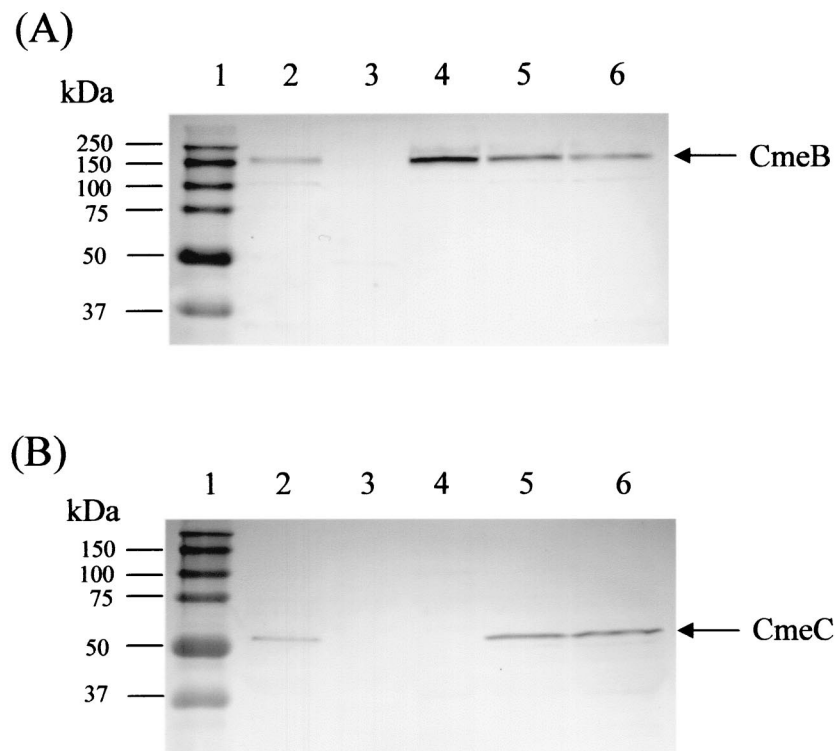


FIG. 1. Immunoblot analysis of CmeB and CmeC expression in wild-type 21190 and various *cmeABC* mutant constructs. Cell envelopes prepared from *C. jejuni* 21190 (lane 2), JL101 (lane 3), JL102 (lane 4), JL103 (lane 5), and JL104 (lane 6) were blotted with specific antibodies against CmeB (A) and CmeC (B). Similar amounts of total proteins were loaded in each lane. Prestained molecular mass markers (lane 1; Bio-Rad) were coelectrophoresed and blotted to allow estimation of the sizes of the proteins.

the plasmid, as shown in Fig. 1. Since the *cmeB* and *cmeC* mutations did not affect the expression of the chromosome copy of *cmeA* in JL101 (28) or JL102 (data not shown), pCME provided an ideal vector for the complementation of the mutants in this study. Electroporation of pCME into JL101 and JL102 created JL103 and JL104, respectively (Table 1). Expression of *cmeB* and *cmeC* in JL103 and JL104 was confirmed by immunoblotting using anti-CmeB and anti-CmeC antibodies as described previously (28).

Susceptibility tests. The MICs of different bile salts, fatty acids, and detergents for *C. jejuni* 21190 and its *cmeABC* mutant constructs were determined using a microtiter broth dilution method as described in a previous publication (28). The compounds utilized in these assays were purchased from Sigma Chemical Co. (cholic acid, chenodeoxycholic acid, taurocholic acid, deoxycholic acid, dehydrocholic acid, glycocholic acid, taurodeoxycholic acid, choleate, capric acid, butyric acid, palmitic acid, and Triton X-100), EM Science (sodium dodecyl sulfate [SDS]), Calbiochem (Empigen BB), and Amresco (Tween 20).

Preparation of chicken intestinal extracts. Ten 21-day-old broiler chickens were used for the preparation of chicken intestinal extracts. All birds tested negative for *Campylobacter* by culturing cloacal swabs. Chicken intestinal extracts were prepared from three different sections of the intestine, including the duodenum, jejunum, and cecum. The intestinal contents from each section were pooled from the 10 chickens. Each pool of chicken intestinal contents was divided in half, and each half was resuspended in the same volume of MH broth or saline by vortex, followed by centrifugation at $10,000 \times g$ at 4°C for 30 min. The supernatant from the MH or saline extraction was sterilized by successive filtration through a series of membrane filters (prefilter 1.2 μm and 0.45 μm ; Millipore Co.). The filtered intestinal extracts were tested for sterility by plating 200 μl of the final filtrate on MH plates and incubating the plates under aerobic and microaerophilic conditions for 3 days, which showed there was no growth of any bacterial colony. The filtered intestinal extracts were used for measuring bile salt concentrations (saline-based extracts) and for in vitro growth inhibition assays (MH-based extracts).

Measurement of total bile salt concentrations. Total bile concentrations in chicken duodenal, jejunal, and cecal extracts were measured using a total bile acids assay kit according to the procedure supplied by the manufacturer (Di-

zyme, San Diego, Calif.). The concentration (expressed as millimolar) of bile salts in each intestinal extract was recorded as the mean of four measurements.

In vitro growth assay. To compare the growth characteristics of *C. jejuni* 21190 and its *cmeABC* mutants in the presence of bile salts and chicken intestinal extracts, an in vitro growth assay was performed in microtiter plates. Each well of the plate contained 225 μl of medium plus 25 μl of *Campylobacter* inoculum (approximately 5×10^6 CFU/ml). Several media were used for the assays, including MH, MH supplemented with sodium choleate (1 mg/ml; ~ 2 mM bile salts), and MH-based chicken intestinal extracts. We chose sodium choleate (Sigma) for the in vitro assay because it is a crude ox bile extract that contains the sodium salts of cholic, glycocholic, deoxycholic, and taurocholic acids, which closely reflect the bile salt components in animal intestine. Triplicate wells were used for each strain and mutant. The cultures were incubated at 42°C under microaerophilic conditions for 48 h. During the incubation, samples were taken at different time points (1, 3, 7, 12, 24, and 48 h postinoculation), serially diluted, and plated on MH agar for enumeration of *Campylobacter* colonies in each sample. The \log_{10} -transformed CFU for each sample was used to compare the growth of various strains and mutants in different media.

Chicken colonization experiments. Newly hatched day-old broiler chickens were obtained from a commercial hatchery. Prior to use, these chicks were screened for *Campylobacter* by culturing cloacal swabs, which were plated onto MH agar plates containing *Campylobacter*-specific growth supplements (SR084E and SR117E; Oxoid). All of the birds tested negative for *Campylobacter*. To compare the wild-type 21190 and its *cmeABC* mutant constructs, 48 2-day-old birds were assigned to four treatment groups (12 birds/group). Each group was inoculated with a similar dose (10^6 CFU/chicken, via oral gavage) of wild-type 21190, JL101, JL102, or JL103. Each group of chickens was maintained in a sanitized wire-floored cage and provided with unlimited access to feed and water. The feed (C-2-88; Ohio Agricultural Research and Development Center, Ohio State University) was manufactured on site and was *Campylobacter*-free and without any animal protein or antibiotic additives. The parent strain and *cmeABC* mutants showed similar growth patterns in the feed extract (data not shown). Thus, the feed should not be a factor influencing the growth of *cmeABC* mutants in the intestine. Because the chickens were given city water, which

TABLE 2. Susceptibilities of strain 21190 and its mutant constructs to different bile salts, fatty acids, and detergents

Antimicrobial	MIC ($\mu\text{g/ml}$)				
	21190	JL101 ^a	JL102 ^a	JL103 ^a	JL104 ^a
Cholic acid	3,125	98 (32)	98 (32)	6,250 (1/2)	6,250 (1/2)
Chenodeoxycholic acid	1,250	19.5 (64)	19.5 (64)	1,250 (—)	1,250 (—)
Taurocholic acid	50,000	780 (64)	780 (64)	50,000 (—)	50,000 (—)
Deoxycholic acid	1,250	9.75 (128)	9.75 (128)	1,250 (—)	1,250 (—)
Dehydrocholic acid	10,000	2,500 (4)	2,500 (4)	10,000 (—)	10,000 (—)
Glycocholic acid	5,000	625 (8)	625 (8)	10,000 (1/2)	10,000 (1/2)
Taurodeoxycholic acid	10,000	78 (128)	78 (128)	10,000 (—)	10,000 (—)
Choleate ^b	6,250	97 (64)	97 (64)	50,000 (1/8)	50,000 (1/8)
SDS	156	39 (4)	39 (4)	312 (1/2)	312 (1/2)
Triton X-100	50,000	24.5 (2,040)	24.5 (2,040)	50,000 (—)	50,000 (—)
Tween 20	50,000	49 (1,020)	49 (1,020)	50,000 (—)	50,000 (—)
Empigen	75	38 (2)	38 (2)	75 (—)	75 (—)
Capric acid	250	31 (8)	31 (8)	250 (—)	250 (—)
Butyric acid	5,000	5,000 (—)	5,000 (—)	5,000 (—)	5,000 (—)
Palmitic acid	>400	>400 (—)	>400 (—)	>400 (—)	>400 (—)

^a The numbers in parentheses indicate the fold differences in MICs between 21190 and its mutant constructs. —, no MIC difference was observed.

^b Choleate (Sigma) is a crude ox bile extract which contains the sodium salts of taurocholic, glycocholic, deoxycholic, and cholic acids.

contained sodium hypochlorite at a concentration of 5 ppm, we also determined if CmeABC influences *Campylobacter* susceptibility to sodium hypochlorite. Wild-type 21190 and *cmeABC* mutants had the same MIC of 262 ppm for sodium hypochlorite, which is well above the chlorine concentration in the city water. In addition, both the wild type and the mutants showed similar survival patterns in fresh drinking water from the chicken house (data not shown). Thus, the feed and drinking water were not factors influencing the survival of *cmeABC* mutants in the chickens.

Fecal samples were collected using cloacal swabs, which were taken every 2 days and diluted in MH broth for enumeration of *Campylobacter* cells. For the groups inoculated with wild-type 21190 and JL103, each fecal suspension was plated onto MH plates containing *Campylobacter*-specific growth supplements (Oxoid) for counting *Campylobacter* colonies. Since inactivation of *cmeABC* makes *Campylobacter* become susceptible to rifampin (28), a selective agent present in the *Campylobacter*-specific growth supplement (SR117E; Oxoid), the standard selective medium was not suitable for the isolation of JL101 and JL102. Therefore, the selective medium was modified by replacing rifampin with kanamycin and retaining the other three selective agents present in the growth supplement, including trimethoprim (10 $\mu\text{g/ml}$), cycloheximide (100 $\mu\text{g/ml}$), and polymyxin B (1 $\mu\text{g/ml}$), which are not substrates of CmeABC (28). This modified selective medium fully supported the growth of JL101 and JL102 in pure culture or spiked fecal samples (data not shown). This modified medium was used for isolation of *Campylobacter* from chickens inoculated with JL101 or JL102.

To determine the minimum infective dose of *C. jejuni* 21190 and JL101, 40 2-day-old broilers were assigned to eight groups (five birds/group), four of which were inoculated with various doses of wild-type 21190 (1.2×10^4 to 1.2×10^7 CFU/chicken). The other four groups were inoculated with different doses of JL101 (3.2×10^6 to 3.2×10^9 CFU/chicken). After inoculation, cloacal swabs were collected from the chickens and cultured for *Campylobacter*. The minimum infective dose was defined as the lowest dose at which at least one chicken of the inoculated group was colonized within 2 weeks after inoculation.

Immunoblotting analysis of CmeABC expression. Expression of *cmeABC* in JL101, JL102, JL103, and JL104 was examined using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described previously (28). Immunoblotting was performed to determine if CmeABC was expressed and immunogenic in vivo. The recombinant proteins for CmeA, CmeB, and CmeC were generated in a previous study (28). The recombinant proteins were separated by SDS-PAGE and were then electrophoretically transferred to a nitrocellulose membrane as described previously (28). The blots were incubated with chicken serum samples (1:100 dilution in blocking buffer), which included three negative control serum samples from 3-week-old *Campylobacter*-free broiler chickens and eight serum samples from 2-year-old specific-pathogen-free layers or 6-week-old broilers experimentally infected with *C. jejuni* (46). After incubation at 25°C for 1 h, the blots were washed three times with phosphate-buffered saline containing 0.05% Tween 20 and subsequently incubated with secondary antibodies (1:1,000 dilution of goat anti-chicken immunoglobulin G-horse radish peroxidase; Kirkegaard & Perry) at 25°C for 1 h. After washing, the blots were developed with the 4 CN membrane peroxidase substrate system (Kirkegaard & Perry).

RESULTS

Effects of *cmeABC* mutations on bile resistance. Our previous study showed that insertional mutation in *cmeB* impaired the expression of both *cmeB* and *cmeC* and increased the mutant's susceptibility to a few selected bile salts (28). To fully examine the substrate spectrum of CmeABC and the role of the individual member of the efflux pump in the efflux of bile salts, we constructed additional mutant constructs and tested their susceptibilities to various bile salts and detergents. In JL102, the insertional mutation in *cmeC* abolished the production of CmeC but did not affect the expression of *cmeA* (data not shown) and *cmeB* (Fig. 1). Similar to JL101, in which both *cmeB* and *cmeC* were not expressed, JL102 showed significantly increased susceptibilities to all bile salts tested in this study (Table 2). The MICs of bile salts for JL101 and JL102 decreased 128-fold for deoxycholic acid and taurodeoxycholic acid; 64-fold for chenodeoxycholic acid, taurocholic acid, and choleate; 32-fold for cholic acid; 8-fold for glycocholic acid; and 4-fold for dehydrocholic acid. JL101 and JL102 also showed increased susceptibilities to other detergents (Table 2). The same MIC changes for JL101 and JL102 indicated that inactivation of CmeC alone would cause malfunction of the CmeABC pump, further supporting the notion that the three members of CmeABC function together in the efflux of substrates. Notably, MICs of Triton X-100 and Tween 20 decreased more than 1,000-fold in these two mutants. The MIC of capric acid decreased eightfold in the mutants, but the MICs of butyric acid and palmitic acid had no changes, suggesting that CmeABC is not the main pump for the efflux of fatty acids.

Complementation of JL101 and JL102 with pCME completely restored the production of *cmeB* and *cmeC* in JL103 and JL104 (Fig. 1), which showed the wild-type level of resistance to bile salts and other detergents (Table 2). These in vitro results indicate that CmeABC contributes significantly to bile resistance in *Campylobacter*.

Inhibition of *Campylobacter* growth by choleate. To further examine the contribution of CmeABC in bile resistance, we compared the in vitro growth characteristics of 21190 and its

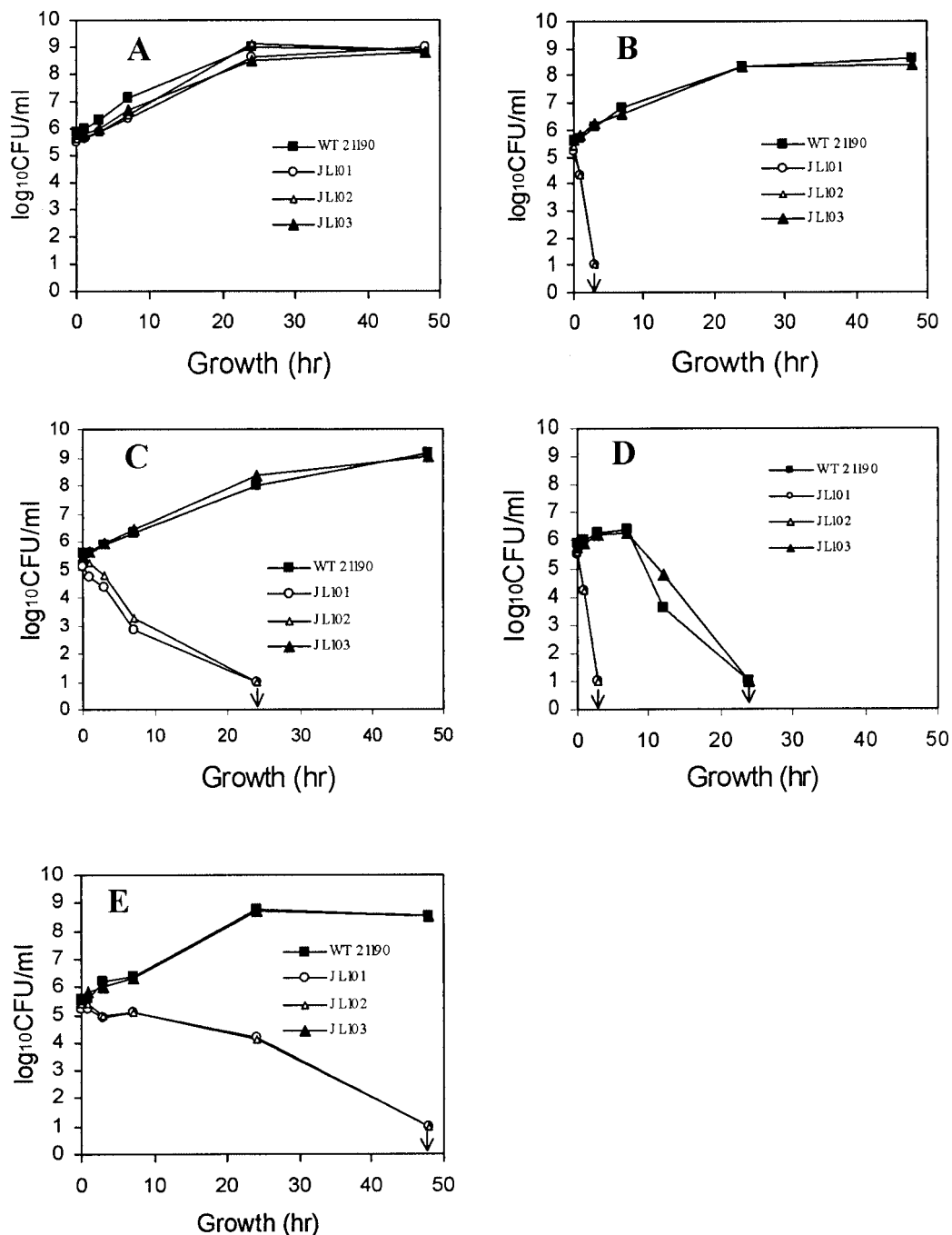


FIG. 2. Effects of bile salts and chicken intestinal extracts on the in vitro growth of *C. jejuni* 21190 and its isogenic *cmeABC* mutants. Bacteria were grown in MH broth (A), MH broth supplemented with sodium cholate (1 mg/ml) (B), chicken duodenal extract (C), chicken jejunal extract (D), and chicken cecal extract (E). The downward arrow indicates that no viable colonies were detected in the experiments, and the detection limit of the method was 10 CFU/ml. Each data point represents the mean value obtained from triplicate wells in the microtiter plate growth assay.

cmeABC mutants in MH broth or MH broth containing 1 mg of cholate/ml (~ 2 mM bile salts). In conventional MH broth, both the wild-type strain and the mutants showed similar growth patterns (Fig. 2A). However, addition of cholate in MH broth greatly inhibited the growth of JL101 and JL102, but it did not have any effect on wild-type 21190 (Fig. 2B). In fact, no viable JL101 and JL102 cells were detected after 3 h of incubation in MH broth with cholate (Fig. 2B). Complemen-

tation of JL101 with pCME (JL103) completely restored the growth of the mutant to the wild-type level in MH broth supplemented with cholate (Fig. 2B). Together, these results indicate that the CmeABC pump is essential for *Campylobacter* growth in bile salts-containing medium in vitro.

Inhibition of *Campylobacter* growth by chicken intestinal extracts. Bile salts are present in animal intestinal contents. To assess if CmeABC was required for the growth of *C. jejuni* in

intestinal extracts, we determined the actual concentrations of bile salts in different sections of chicken intestinal tract and then measured their effects on *Campylobacter* growth. As determined with a commercial kit, total bile concentrations in chicken duodenum, jejunum, and cecum were 3.5 ± 0.2 , 14.0 ± 0.3 , 0.17 ± 0.02 mM, respectively. Based on the estimated average molecular mass of 500 Da for bile salts, the total bile salt concentrations in chicken duodenum, jejunum, and cecum were 1.75, 7, and 0.085 mg/ml, respectively. These measurements were comparable to the results reported in a previous study in which bile salt concentrations in chicken intestinal extract were determined using a procedure involving bile salt extraction followed by enzymatic analysis (14). Although varied in different locations of the intestine, bile salt concentrations, particularly in the duodenum and jejunum, are far above the MICs of bile salts for *cmeABC* mutants (Table 2).

To determine if chicken intestinal extracts inhibited the growth of *Campylobacter*, wild-type 21190, JL101, JL102, and JL103 cells were grown in MH-based intestinal extracts. As shown in Fig. 2, the growth of JL101 and JL102 was greatly inhibited compared with that of wild-type 21190, although each intestinal extract showed a unique inhibitory pattern on *Campylobacter* growth. The wild-type strain grew normally in chicken duodenal extract as in MH broth, but the mutants did not show any growth. In fact, no viable JL101 and JL102 cells were detected after 24 h of incubation (Fig. 2C). Unlike the duodenal extract, the jejunal extract not only showed a striking bactericidal effect on the mutants but also had strong growth inhibition for wild-type 21190 (Fig. 2D). However, the two mutants experienced much faster and greater growth reductions than the wild-type strain in the jejunal extract. After 3 h of incubation, both JL101 and JL102 were no longer detected, suggesting that the jejunal extract killed the mutants within 3 h. On the contrary, wild-type 21190 did not show any growth reduction for the first 6 h, had 1 log unit of growth reduction by 12 h of incubation, and was below the detection limit by 24 h of incubation (Fig. 2D). In the cecal extract, which had the lowest bile concentration, wild-type 21190 grew normally and reached stationary phase within 24 h (Fig. 2E). However, JL101 and JL102 failed to grow in the cecal extract, although both of them survived longer than in the duodenal extract. As shown with JL103, complementation of the *cmeB* mutant with pCME fully restored the growth of the mutant to the wild-type level in each of the three intestinal extracts (Fig. 2). Additional experiments conducted at different times also showed similar growth patterns for 21190 and its *cmeABC* mutants as described above (data not shown). In addition, the *cmeABC* mutants of strain 81-176 also showed growth defects in the chicken intestinal extracts (data not shown). The overall inhibitory effect of each intestinal extract on *Campylobacter* growth was well correlated with the bile concentration in each preparation. These results clearly demonstrated that CmeABC is essential for *Campylobacter* survival and growth in chicken intestinal extracts.

CmeABC is essential for the colonization of *Campylobacter* in chickens. The inability of JL101 and JL102 to grow in the intestinal extracts suggested that CmeABC may be required for *Campylobacter* adaptation in chickens. To test this possibility, a typical chicken colonization model was used to determine the role of CmeABC in the colonization of *C. jejuni*. As shown in

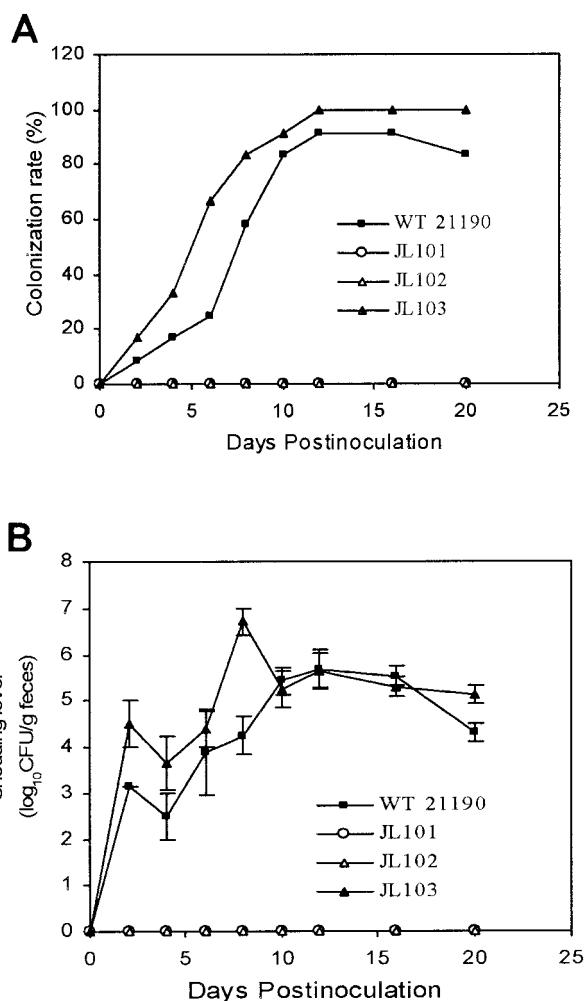


FIG. 3. Colonization of *C. jejuni* 21190 and its isogenic *cmeABC* mutants in chickens. (A) Percentage of chickens colonized by *C. jejuni* after inoculation. (B) The shedding level of *Campylobacter* in chickens colonized by *C. jejuni* after inoculation. Each data point represents the mean CFU of the colonized chickens in each group. Standard errors are indicated by error bars.

Fig. 3, wild-type 21190 colonized the chickens as early as 2 days postinoculation, and the majority of chickens were colonized at day 8 postinoculation as determined by culturing cloacal swabs. In individual chickens, the density of the organism was as high as 10^7 CFU/g of feces. In contrast, *C. jejuni* was not detected throughout the study in any of the cloacal samples collected from the chickens inoculated with JL101 or JL102 (Fig. 3). The construct JL103 displayed a colonization rate and shedding level comparable with those of wild-type 21190 (Fig. 3).

To further compare the difference between wild-type 21190 and JL101 in colonization of chickens, the minimum infective dose was determined using multiple groups of chickens. As shown in Table 3, wild-type 21190 was able to colonize some of the chickens at a dose as low as 1.2×10^5 CFU/chicken, while JL101 failed to colonize the chickens even at a dose as high as 3.2×10^9 CFU/chicken (Table 3). At day 13 after the inoculation, the chickens inoculated with the highest dose of JL101 were necropsied and culturing of the cecal contents revealed no *Campylobacter* in the cecum, further confirming that JL101

TABLE 3. Determination of the minimum infective dose of 21190 and JL101 in broiler chickens

Strain	Dose (CFU/chicken)	% Chickens colonized on day after inoculation:							
		0	1	2	4	6	8	11	13
WT 21190	1.2×10^4	0	0	0	0	0	0	0	0
	1.2×10^5	0	0	0	0	0	0	40	20
	1.2×10^6	0	20	20	40	60	60	80	60
	1.2×10^7	0	40	60	60	80	80	100	100
JL101	3.2×10^6	0	0	0	0	0	0	0	0
	3.2×10^7	0	0	0	0	0	0	0	0
	3.2×10^8	0	0	0	0	0	0	0	0
	3.2×10^9	0	0	0	0	0	0	0	0 ^a

^a Chickens were necropsied and cecal contents were cultured for *Campylobacter*.

failed to colonize the chicken intestinal tract. Thus, the minimum infective dose for JL101 was at least 2.6×10^4 -fold higher than that of wild-type 21190. The difference between JL101 and 21190 in colonizing chickens was not attributable to the growth rate, because the in vitro growth characteristics of JL101 were quite similar to those of wild-type 21190 in conventional MH broth (Fig. 2A). Together, these results strongly indicated that CmeABC is essential for the in vivo adaptation of *C. jejuni* in the intestinal tract.

To confirm that the isolates recovered from the experimental chickens were derived from the inoculated 21190 or its derivatives, the *cmp* gene encoding the major outer membrane protein was PCR amplified using primers F3 and R3 (52) from representative *Campylobacter* isolates obtained from the chickens. The sequence data showed that the *cmp* sequence was identical to that of 21190 (data not shown). In addition, the recovered JL103 isolates were confirmed by detecting the chloramphenicol resistance marker carried by pCME. These results indicated that no environmental contamination of the chickens by *Campylobacter* occurred in the experiments.

CmeABC is expressed and immunogenic in vivo. To determine if CmeABC is expressed and immunogenic in vivo, immunoblotting was performed using purified recombinant CmeABC peptides and serum samples from chickens infected with *Campylobacter*. As shown in Fig. 4A, the serum from a *Campylobacter*-infected chicken showed positive reaction to recombinant CmeA, CmeB, and CmeC, while the serum from a *Campylobacter*-free chicken did not react with any of the three recombinant proteins. Furthermore, multiple chicken sera were tested for reactivity with recombinant CmeC (Fig. 4B). All eight sera from *Campylobacter*-infected chickens showed a vivid antibody reactivity to recombinant CmeC (Fig. 4B), which is a predicted outer membrane component of the CmeABC efflux system. All the sera from *Campylobacter*-free chickens were negative for CmeC (Fig. 4B, lanes 11 to 13). These results suggest that CmeABC is expressed during *Campylobacter* infection of chickens and elicits a specific antibody response in the host.

DISCUSSION

The results of this study clearly demonstrated that CmeABC, by mediating bile resistance, plays a critical role in the in vivo

adaptation of *Campylobacter* in chickens. This conclusion is supported by the following evidence. Firstly, CmeABC contributes significantly to *Campylobacter* resistance to various bile salts and detergents as determined using site-directed mutagenesis (Table 2). Secondly, mutations in *cmeABC* resulted in growth defects in bile salts-containing media and chicken intestinal extracts (Fig. 2). Thirdly, inactivation of *cmeABC* abolished the ability of *Campylobacter* to colonize chickens (Fig. 3). These phenotypic changes associated with the *cmeABC* mutants were directly linked to the inactivation of *cmeABC*, because the insertional mutation did not cause a polar effect on the gene (*Cj0364*) downstream of *cmeABC* (28) and complementation of the mutations with pCME fully restored the growth in bile-containing media and in vivo colonization (Fig. 2 and 3). These findings plus the fact that CmeABC is expressed during the course of *Campylobacter* colonization of the chicken host (Fig. 4) strongly indicate that CmeABC is essential for successful colonization of *C. jejuni* in the intestinal environment.

Bile salts are detergent-like amphipathic molecules normally present in animal intestinal tracts. As surface-active detergents, bile salts have potent antimicrobial activity (16, 20, 21). The major species of bile salts produced by the liver are the taurine and glycine conjugates of cholic acid and chenodeoxycholic acid, which are known as primary bile salts (2). These bile salts undergo further structural modifications (e.g., deconjugation and dehydroxylation) and result in various bile acid metabolites (e.g., cholic acid, chenodeoxycholic acid), which are known as secondary and tertiary bile salts (2). Under conditions existing in the gut of humans and animals, the bile concentration can be as high as 23 mM in jejunum, although a concentration of 2 mM is generally considered to be necessary for the formation of micelles for lipid digestion (10, 14, 51). The high concentration of bile salts in chicken small intestine (duodenum and jejunum) constitutes a formidable barrier for *Campylobacter*, and even the wild-type 21190 experienced a significant reduction in viability after 12 h of incubation in the jejunal extract (Fig. 2). Although the filtrates of chicken intestinal contents may not represent the microenvironment in which *C. jejuni* lives in the chicken intestinal tract, the inhibitory effect of the jejunal extract on *Campylobacter* growth is consistent with the fact that *Campylobacter* is rarely isolated from the small intestine of chickens (1, 6). In the cecum, the predominant site for *Campylobacter* colonization in chickens, the bile concentration is low enough to allow the growth of wild-type *Campylobacter* and high enough to inhibit the growth of *cmeABC* mutants (Fig. 2). This in vitro observation was consistent with the in vivo result that mutants JL101 and JL102 failed to colonize the intestinal tract (Fig. 3 and Table 3). Together, these findings strongly indicate that *Campylobacter* needs a functional CmeABC for growth in the gut.

To determine the substrate spectrum of the CmeABC system, we also investigated the role of CmeABC in the resistance to nonbile detergents as well as hydrophobic fatty acids. Strikingly, mutations in *cmeABC* resulted in much greater MIC reductions for nonionic detergents (Triton X-100 and Tween 20) than those for an ionic detergent (SDS) or zwitterionic detergent (Empigen). The susceptibility test conducted in this study revealed that CmeABC has a limited role in the efflux of fatty acids, suggesting that fatty acid resistance is an unlikely

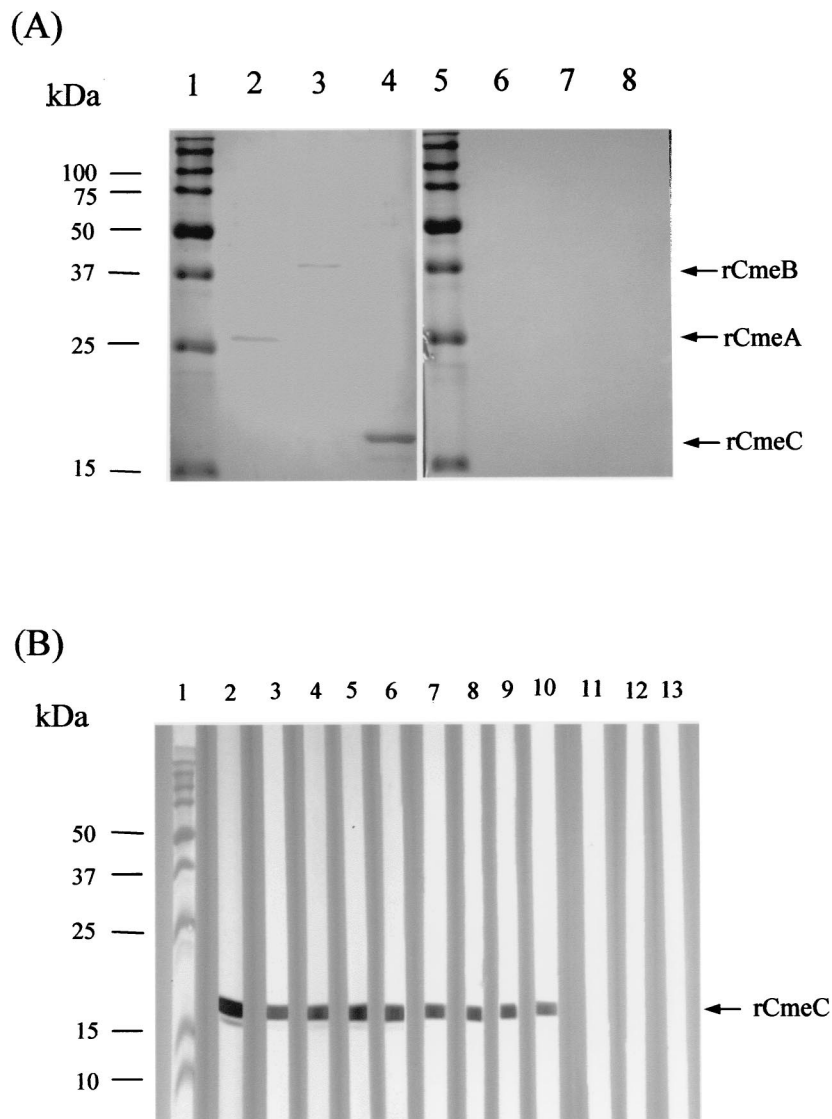


FIG. 4. Immunoblot analysis of in vivo antibody responses to CmeABC. (A) Purified recombinant CmeA (lanes 2 and 6), CmeB (lanes 3 and 7), and CmeC (lanes 4 and 8) peptides were blotted with serum from a chicken infected with *C. jejuni* (lanes 1 to 4) or serum from a *Campylobacter*-free chicken (lanes 5 to 8). (B) The recombinant CmeC was blotted with the rabbit anti-CmeC antibody (lane 2) or with individual chicken serum samples (lanes 3 to 13). Lanes 3 to 6, serum samples from 2-year-old *Campylobacter*-infected specific-pathogen-free layers; lanes 7 to 10, serum samples from 6-week-old broiler chickens infected with *C. jejuni*; lanes 11 to 13, serum samples from 3-week-old broiler chickens which were free of *Campylobacter*. Prestained molecule mass markers (lanes 1 and 5 in panel A and lane 1 in panel B; Bio-Rad) were coelectrophoresed and blotted to allow estimation of the sizes of the proteins.

mechanism by which CmeABC contributes to *Campylobacter* adaptation in vivo. It should be pointed out that the MIC changes observed in JL101 and JL102 were not attributable to pH. All fatty acids and bile salts used in this study are sodium salts, and their pH is approximately 7.0 after solubilization in MH broth for the MIC test (data not shown). In addition, wild-type 21190 and JL101 grew similarly in acidic MH medium (pH 5.0), suggesting that CmeABC is not involved in resistance to acidic stress.

The chicken is a natural host and major reservoir for *C. jejuni*. This organism is well adapted to the intestinal tract of poultry, which provides an excellent model for evaluating *Campylobacter* colonization (not a model for disease). Colo-

nization of chickens by *C. jejuni* occurs mainly in lower intestines, where the organism mainly infects cecal and cloacal crypts (1, 6, 33). Unlike the infection in mammals (e.g., mice, swine, rabbits, monkeys, and humans), in which *C. jejuni* may invade intestinal epithelial cells and cause histopathologic changes (3, 8, 44, 45), infection of chickens by *C. jejuni* does not result in invasion of the intestinal epithelium and clinical disease under normal conditions (6, 33). Newly hatched chickens are usually *Campylobacter*-free, eliminating the potential contamination of experimental chickens by unknown *Campylobacter* strains. Using this chicken model, we demonstrated the essential role of CmeABC in in vivo colonization. To exclude the possibility that the colonization deficiency of *cmeABC* mu-

tants was due to factors that are not related to bile resistance, we evaluated bacterial growth in feed extract and drinking water (detailed in Materials and Methods). Both wild-type 21190 and *cmeABC* mutants showed similar growth and survival patterns in the feed and drinking water. These observations further support our conclusion that the key role of CmeABC in *Campylobacter* colonization is related to bile resistance.

Efflux of antibiotics by MDR pumps is considered an opportunistic function of the pumps, and the natural functions of MDR efflux systems are still largely unknown (36, 39, 40). Previous studies using *in vitro* assays indicated that AcrAB of *Escherichia coli* (12, 32, 48), MtrCDE of *Neisseria gonorrhoeae* (17), and VceAB of *Vibrio cholerae* (9) are involved in the resistance to bile and/or fatty acids, suggesting that these MDR pumps may provide a protective role in the survival of pathogens in the gut or other mucous sites. However, these studies lack direct evidence showing the role of efflux pumps in the *in vivo* adaptation of these pathogenic bacteria. Work presented in this study provides direct evidence showing the *in vivo* protective function of CmeABC by mediating bile resistance. According to this observation plus our previous finding (28) that CmeABC is broadly distributed and expressed in *Campylobacter* strains from various sources, it can be concluded that bile resistance is an important natural function for CmeABC. Despite the constitutive expression of CmeABC in wild-type *C. jejuni* strains, it is possible that CmeABC is subject to regulation by other factors. This speculation is based on the finding of a putative transcriptional repressor gene occurring immediately upstream of *cmeA* and the presence of a putative operator in the intergenic region between *cmeA* and its upstream open reading frame (28). However, immunoblotting analysis of *Campylobacter* cells grown in the presence or absence of cholic acid did not reveal obvious differences in the expression of CmeABC (data not shown). At this stage, it is unknown if CmeABC could be regulated by other bile salts or other substrates. Investigation of the regulatory mechanism of CmeABC will further improve our understanding of the function of CmeABC in the adaptation of *Campylobacter* in various niches.

It has been proposed that inhibiting MDR efflux systems is one approach to enhance drug accumulation inside the bacterial cell, thereby increasing bacterial susceptibility to antimicrobials (30, 41). Recently, MDR efflux pump inhibitors have been developed and demonstrated to potentiate the activity of antimicrobial agents against a range of gram-negative bacteria (29, 30). The presence of such inhibitors also resulted in a decreased frequency of emergence of *Pseudomonas aeruginosa* strains that are highly resistant to fluoroquinolones (29). Based on the findings from this study, we speculate that inhibitors targeting the CmeABC efflux pump may not only control antibiotic resistance but also increase the susceptibility of *C. jejuni* to *in vivo* bile salts, consequently decreasing the colonization level of *Campylobacter*. Such pump inhibitors could be directly used as novel antimicrobials for therapeutic intervention of *Campylobacter* infection. Inhibiting bacterial efflux of bile salts with pump inhibitors in the gut may be a general approach for developing therapeutic measures for enteric pathogens. From the standpoint of vaccine development, the outer membrane components of MDR pumps of gram-negative bacteria may be

exploited as the targets of immune interventions, preventing the development of antibiotic resistance and the establishment of *in vivo* infection (27). In this study, we demonstrated that the outer membrane component CmeC is expressed and immunogenic *in vivo*, supporting the feasibility of targeting CmeC for immune protection against *Campylobacter* colonization. This possibility remains to be examined in future studies.

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