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Abstract

CmeABC, a multidrug efflux pump, is involved in the resistance of *Campylobacter jejuni* to a broad spectrum of antimicrobial agents and is essential for *Campylobacter* colonization in animal intestine by mediating bile resistance. Previously, we have shown that expression of this efflux pump is under the control of a transcriptional repressor named CmeR. Inactivation of CmeR or mutation in the *cmeABC* promoter (P_{cmeABC}) region derepresses *cmeABC*, leading to overexpression of this efflux pump. However, it is unknown if the expression of *cmeABC* can be conditionally induced by the substrates it extrudes. In this study, we examined the expression of *cmeABC* in the presence of various antimicrobial compounds. Although the majority of the antimicrobials tested did not affect the expression of *cmeABC*, bile salts drastically elevated the expression of this efflux operon. The induction was observed with both conjugated and unconjugated bile salts and was in a dose- and time-dependent manner. Experiments using surface plasmon resonance demonstrated that bile salts inhibited the binding of CmeR to P_{cmeABC}, suggesting that bile compounds are inducing ligands of CmeR. The interaction between bile salts and CmeR likely triggers conformational changes in CmeR, resulting in reduced binding affinity of CmeR to P_{cmeABC}. Bile did not affect the transcription of *cmeR*, indicating that altered expression of *cmeR* is not a factor in bile-induced overexpression of *cmeABC*. In addition to the CmeR-dependent induction, some bile salts (e.g., taurocholate) also activated the expression of *cmeABC* by a CmeR-independent pathway. Consistent with the elevated production of CmeABC, the presence of bile salts in culture media resulted in increased resistance of *Campylobacter* to multiple antimicrobials. These findings reveal a new mechanism that modulates the expression of *cmeABC* and further support the notion that bile resistance is a natural function of CmeABC.

Disciplines

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Bile Salts Modulate Expression of the CmeABC Multidrug Efflux Pump in *Campylobacter jejuni*

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CmeABC, a multidrug efflux pump, is involved in the resistance of *Campylobacter jejuni* to a broad spectrum of antimicrobial agents and is essential for *Campylobacter* colonization in animal intestine by mediating bile resistance. Previously, we have shown that expression of this efflux pump is under the control of a transcriptional repressor named CmeR. Inactivation of CmeR or mutation in the *cmeABC* promoter (P_{cmeABC}) region derepresses *cmeABC*, leading to overexpression of this efflux pump. However, it is unknown if the expression of *cmeABC* can be conditionally induced by the substrates it extrudes. In this study, we examined the expression of *cmeABC* in the presence of various antimicrobial compounds. Although the majority of the antimicrobials tested did not affect the expression of *cmeABC*, bile salts drastically elevated the expression of this efflux operon. The induction was observed with both conjugated and unconjugated bile salts and was in a dose- and time-dependent manner. Experiments using surface plasmon resonance demonstrated that bile salts inhibited the binding of CmeR to P_{cmeABC}, suggesting that bile compounds are inducing ligands of CmeR. The interaction between bile salts and CmeR likely triggers conformational changes in CmeR, resulting in reduced binding affinity of CmeR to P_{cmeABC}. Bile did not affect the transcription of *cmeR*, indicating that altered expression of *cmeR* is not a factor in bile-induced overexpression of *cmeABC*. In addition to the CmeR-dependent induction, some bile salts (e.g., taurocholate) also activated the expression of *cmeABC* by a CmeR-independent pathway. Consistent with the elevated production of CmeABC, the presence of bile salts in culture media resulted in increased resistance of *Campylobacter* to multiple antimicrobials. These findings reveal a new mechanism that modulates the expression of *cmeABC* and further support the notion that bile resistance is a natural function of CmeABC.

Multidrug efflux systems (often named multidrug resistance [MDR] pumps), particularly the efflux pumps belonging to the resistance-nodulation-cell division (RND) superfamily, contribute significantly to bacterial resistance to antimicrobial compounds, including those (e.g., bile salts) naturally present in animal hosts (12, 22, 36, 39). The expression of MDR efflux pumps is usually modulated by transcriptional regulators (10), many of which are local repressors that interact directly with the promoters of the genes encoding efflux pumps. Mutations in the repressors or repressor-binding sequences can result in overexpression of efflux pumps, which can increase bacterial resistance to structurally unrelated antimicrobial agents (10, 18, 36, 39). In addition to the mutation-based mechanisms that result in sustained overexpression of MDR efflux pumps in bacteria, the expression of some MDR efflux pumps can be conditionally induced by the substrates of these pumps (10, 36). This induction is usually mediated by the direct interaction of the substrates with repressor molecules, leading to reduced binding of repressors to operator DNA and increased transcription of efflux genes. Since sustained overexpression of efflux pumps can be detrimental to bacterial fitness (7, 26), conditional induction of efflux machineries by their substrates provides bacterial pathogens with a cost-efficient mechanism for rapid adaptation to environmental changes.

CmeABC is characterized as an RND-type MDR pump in *Campylobacter jejuni* (21, 38), the leading bacterial cause of food-borne enteritis in humans in many industrialized countries (9). As an enteric pathogen, *Campylobacter* has evolved multiple mechanisms to adapt to the environment in the gastrointestinal tract as well as clinical antimicrobial treatments. One of the major mechanisms utilized by *C. jejuni* for adaptation is CmeABC, which is encoded by a three-gene operon and acts synergistically with other mechanisms in conferring intrinsic and acquired resistance to structurally diverse antimicrobials (21, 25, 38). Notably, CmeABC plays a key role in mediating bile resistance and is essential for *Campylobacter* growth in bile-containing media and in animal intestinal tract, as evidenced by the inability of a *cmeB* null mutant to colonize chickens (22). As an important efflux mechanism, *cmeABC* is constitutively expressed at a moderate level in wild-type *Campylobacter* strains cultured in conventional media (21, 34). This moderate-level expression of *cmeABC* is controlled by a transcriptional repressor named CmeR (19). CmeR is encoded by the gene (named *cmeR*) located immediately upstream of

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

Plasmid or strain	Description	Source or reference
Plasmids		
pMW10	*E. coli-C. jejuni* shuttle vector with promoterless *E. coli lacZ* gene, Kan^r	51
pIT81	pMW10 derivative with the *cmeABC* promoter of wild-type *C. jejuni* 81-176 inserted upstream of *lacZ*	19
Strains		
81-176	Wild type; isolated from human	2
JL108	81-176 containing pMW10	19
JL110	81-176 containing pIT81	19
JL111	JL110 derivative; *cmeR*::*cm*	19
JL112	JL110 derivative; *cbrR*::*cm*	This study

cmeABC and is a member of the TetR family of transcriptional regulators. Like the N-terminal region of other members in the TetR family, the N-terminal region of CmeR contains a typical DNA-binding α-helix-turn-α-helix (HTH) motif, while the C-terminal region is predicted to be involved in the interaction with inducers. *cmeR* is transcribed in the same direction as *cmeABC*, and the intergenic region between *cmeR* and *cmeA* contains the promoter for *cmeABC* (P_{cmeABC}). As a transcriptional factor, CmeR directly binds to the inverted repeat in P_{cmeABC} and represses the transcription of *cmeABC*. Inactivation of CmeR or mutation in the promoter sequence impedes the repression and leads to enhanced production of the MDR efflux pump in *C. jejuni* (19).

Despite the recent progress in understanding the function and regulation of CmeABC, it is not known if expression of *cmeABC* is inducible by the substrates it extrudes. Given that CmeABC plays a significant role in antimicrobial resistance and *Campylobacter* colonization of the intestinal tract, inducible expression of *cmeABC* may facilitate rapid adaptation of *Campylobacter* to environmental changes. In this study, we showed that most substrates of CmeABC, including various antibiotics, did not affect *cmeABC* expression. However, both conjugated and nonconjugated bile salts dramatically induced the expression of *cmeABC*. This induction is mediated by inhibition of CmeR binding to P_{cmeABC} as well as a CmeR-independent activation pathway. These findings reveal a new mechanism that modulates *cmeABC* expression in response to the presence of bile in the environment and further support the notion that bile resistance is the natural function of CmeABC.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Various *Campylobacter* strains, mutants, and plasmids used in this study and their sources are listed in Table 1. These isolates were routinely grown in Mueller-Hinton (MH) broth (Difco) or agar at 42°C under microaerobic conditions, which were generated using a *Campy*gene (Oxoid) gas pack in enclosed jars. When needed, MH media were supplemented with kanamycin (30 μg/ml), chloramphenicol (4 μg/ml), or various concentrations of bile salts.

Chemical compounds. The compounds used for the *cmeABC* induction assays and the antimicrobial susceptibility tests were purchased from Sigma Chemical Co. (norfloxacin, tetracycline, ampicillin, cefotaxime, rifampin, erythromycin, fusidic acid, novobiocin, sodium salicylate, chloramphenicol, cholate, deoxycholate, taurodeoxycholate, glycocholate, chenodeoxycholate, choleate, and taurocholate), ICN Biomedicals, Inc. (ciprofloxacin), and AMRESCO (ethidium bromide). All bile compounds are sodium salts, and their pH is approximately 7.0 after solubilization in MH broth. Choleate is a crude ox bile extract which contains the sodium salts of taurocholic, glycocholic, deoxycholic, and cholic acids.

Antimicrobial susceptibility tests. The MICs of different antimicrobials in *C. jejuni* 81-176 were determined using a microtiter broth dilution method as described in our previous publications (21, 22). To determine the effect of bile salts on the susceptibility of *C. jejuni* 81-176 to other antimicrobials, MH broth was supplemented with cholate or taurocholate to final concentrations of 1 mg/ml and 25 mg/ml, respectively. The concentrations of the two bile salts were chosen according to their known MICs in strain 81-176 (21, 22) and were sublethal (0.5× MICs) to *Campylobacter* growth. Three independent experiments were conducted to confirm the reproducibility of the MIC data.

Construction of JL112. The *cbrR* mutant of *C. jejuni* F38011 (40) was provided by Michael E. Konkel (Washington State University). Genomic DNA was extracted from the *cbrR* mutant and was used to transform JL110 (Table 1) by the method of natural transformation as described by Wang and Taylor (50). This transformation procedure introduced the *cbrR* null mutation into JL110, creating mutant JL112 (Table 1). The *cbrR* mutation in JL112 was confirmed by PCR.

Assays of *cmeABC* transcription under different conditions. Transcription of *cmeABC* was determined by measuring β-galactosidase (LacZ) activity in the *Campylobacter* strains harboring the P_{cmeABC}-*lacZ* transcriptional fusion as described in our previous study (19). Briefly, the strains containing the reporter plasmid were grown for 16 h to log phase (absorbance at 600 nm, approximately 0.2) in MH broth with or without sublethal concentrations (0.5× MICs) of antimicrobials, including bile salts. *Campylobacter* cultures were washed twice in cold phosphate-buffered saline, and the β-galactosidase in each culture was measured as described by Miller (30). To determine if induction of *cmeABC* by bile is time dependent, 100 ml of log-phase culture was equally divided into two portions and inducer cholate was added into one part to a final concentration of 1 mg/ml. At different time points, 1 ml each of the induced and noninduced cultures was taken for β-galactosidase activity as described above. For each time point, triplicate samples were collected for measurements. For measuring dose-dependent induction, strain 81-176 was grown in MH broth with various concentrations (0, 0.125, 0.25, 0.5, 1, and 2 mg/ml) of cholate for 16 h, and then the cells were collected for β-galactosidase activity.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. To prepare whole cell lysates, *C. jejuni* 81-76 was grown to late log phase ($\sim 2 \times 10^9$ cells/ml) in MH broth without or with different concentrations (1 and 2 mg/ml) of cholate, harvested by centrifugation, and adjusted by using phosphate-buffered saline to the same absorbance (0.230) at 600 nm. Approximately 4×10^8 whole cells were loaded in each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a 12% (wt/vol) polyacrylamide separating gel (17). Production of CmeB and CmeC was examined by immunoblotting using anti-CmeB and anti-CmeC antibodies as described previously (21).

Analysis of CmeR interaction with P_{cmeABC} by SPR. Surface plasmon resonance (SPR) is a refractometry-based technique that allows the measurement of biomolecular interactions in real time as changes of mass concentrations on a sensor surface (41). A Biacore 1000 (Biacore AB, Uppsala, Sweden) was used in this study to examine the interaction of CmeR with P_{cmeABC} in the presence or absence of bile salts. Recombinant CmeR of strain 81-176 was produced in *Escherichia coli* as described in a previously published work (19). DNA probes corresponding to P_{cmeABC} (nucleotides −100 to −1 upstream of the ATG start codon of *cmeA*) and an internal *cmeA* gene fragment (nucleotides 217 to 316) of strain 81-176 were synthesized by Sigma (St-Quentin-Fallavier, France). The internal *cmeA* fragment was used as a negative control for nonspecific binding (19). The probes were immobilized onto streptavidin-coated sensor chips, which were supplied by Biacore AB (Uppsala, Sweden). Before immobilization, the biosensor surface was washed three times by injecting 20 μl of 1 M NaCl, 50 mM NaOH at a flow rate of 20 μl/min as indicated by the manufacturer. Immobilization was performed at a flow rate of 5 μl/min by using Tris-HCl (10 mM Tris-HCl, 300 mM NaCl, 0.5 mM EDTA, pH 7.1) as the running buffer. First, a 5′-biotinylated single-stranded promoter DNA or the control DNA was resuspended in the Tris-HCl buffer, denatured at 65°C for 2 min, and then injected (0.62 μg/ml, 35 μl) onto the streptavidin-coated dextran. The predenatured (65°C for 2 min) cDNA strand was subsequently injected (55 μg/ml, 35 μl). Hybridization between the complementary strands formed double-stranded DNA probes immobilized onto the sensor chip with a surface density of 700 resonance units (RU). Following the DNA immobilization, the coated surface was washed by two injections (5 μl) of regenerating buffer (10 mM Tris-HCl, 2 M NaCl, pH 8.0). The specific interaction of CmeR with the promoter DNA was examined by injecting 30 μl of recombinant CmeR at various concentrations (13 to 208 nM) in running buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.5

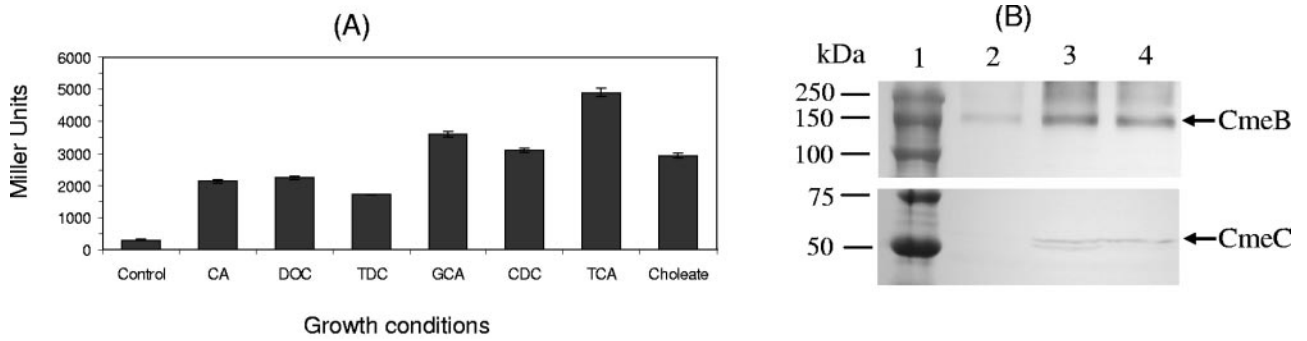

FIG. 1. Induction of *cmeABC* expression by bile salts in *C. jejuni* 81-176. (A) Effects of various bile salts on the transcription of *cmeABC* in 81-176, as measured by transcriptional fusion (JL110). For the LacZ assay, JL110 was grown in the absence (control) of bile salt or presence of the following bile salts at the concentrations sublethal to *Campylobacter*: 2 mg/ml of cholate (CA), 2 mg/ml of deoxycholate (DOC), 2.5 mg/ml of taurodeoxycholate (TDC), 5 mg/ml of glycocholate (GCA), 3 mg/ml of chenodeoxycholate (CDC), 25 mg/ml of taurocholate (TCA), and 2.5 mg/ml of choleate. The bars represent the means ± standard deviations of triplicate samples from a single representative experiment. (B) Immunoblot analysis of CmeB and CmeC production in 81-176 grown in the absence (lane 2) or presence of cholate with final concentrations of 2 mg/ml (lane 3) and 1 mg/ml (lane 4). The same number of bacterial cells (based on optical density at 600 nm) was loaded in each lane. Prestained protein molecular mass markers (Bio-Rad) are shown in lane 1. The positions of CmeB and CmeC are indicated by arrows. Two different blots, immunostained with anti-CmeB (top) and anti-CmeC (bottom) antibodies, are shown.

mM EDTA, 0.005% P20, pH 8.0) at a flow rate of 20 μl/min. Association was monitored during sample injection, and dissociation was observed during washing with the running buffer.

Prior to the inhibition experiments with bile salts, injection of sodium choleate in the concentration range (2 to 125 g/ml) was first tested alone to determine if the bile salts affected the stability of DNA immobilized on the streptavidin-coated sensor chips. In every case, the decrease of signals was less than 5 RU, indicating that bile salts did not have a significant impact on the immobilized DNA. Two distinct experiments to determine the effect of sodium choleate on the binding of CmeR to P_{cmeABC} were performed. In experiment 1, CmeR (104 nM) was incubated at 25°C for 5 min with sodium choleate at various concentrations (2 to 125 μg/ml) before injection, while in experiment 2 various concentrations of sodium choleate (2 to 125 μg/ml, 60 μl) were injected after CmeR (104 nM) bound to DNA. Each assay was also performed under the same conditions on the control flow cell coated with the *cmeA* internal fragment for nonspecific-binding subtraction. Kinetic data were analyzed using BIA evaluation software (version 3.1). The calculated kinetic constants (k_{on} and k_{off}) were validated with the chi-square test by using the 1:1 Langmuir model. The dissociation constant (K_D) is calculated as the ratio of k_{off}/k_{on}. Before analysis, all binding curves were corrected for nonspecific background and bulk refractive index by subtracting the reference curve obtained from the control flow cell.

Real-time quantitative RT-PCR analysis of *cmeR* transcription in the presence of bile salts. *C. jejuni* 81-176 was grown in MH broth or MH broth with cholate (final concentrations of 0.5, 1, and 2 mg/ml) for 16 h under microaerobic conditions. At harvest, 2 volumes of RNAprotect bacterial reagent (QIAGEN, Valencia, CA) were added to the cultures to stabilize total bacterial RNA and the mixtures were incubated at room temperature for 5 min. Then, the bacterial cells were collected by centrifugation at 5,000 × *g* for 10 min. Total RNA in each sample was isolated by using an RNasy mini kit (QIAGEN) according to the manufacturer's instructions. Isolated RNA was further treated with TURBO DNA-free (Ambion) to remove DNA contamination. *cmeR*-specific primers (F3, 5′-ATTTTCAATCAACCAGAAGCTG-3′, and R3, 5′-TCCAATTGGCAAGA TGTCTATC-3′) and primers specific for the *Campylobacter* 16S RNA gene (16SF, 5′-TACCTGGGCTTGATATCCTA-3′, and 16SR, 5′-GGACTTAACCC AACATCTCA-3′) were designed using the Primer3 online interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each amplicon was analyzed with the MFold server (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/) to avoid secondary RNA structures and hairpin loops. Before being used for quantitative reverse transcriptase PCR (RT-PCR), each RNA template and each primer set were tested with a conventional one-step RT-PCR kit and a regular PCR kit (Invitrogen) to ensure specific amplification from the target mRNA and no detectable DNA contamination in the RNA preparation. For each RNA template, to generate the standard curve for quantification of the target transcript, 10-fold dilution series between 2.5 pg and 25 ng were made for each RNA template and used for RT-PCR with the MyiQ iCycler real-time PCR detection system (Bio-Rad, CA). The RT-PCRs were conducted using the iScript one-step RT-PCR kit with SYBR green (Bio-Rad). Triplicate reactions in a volume of 20 μl were performed for each dilution of the RNA template. Thermal cycling conditions were as follows: 10 min at 50°C, 5 min at 60°C followed by 5 min at 95°C, and then 40 cycles of 10 s at 95°C and 30 s at 55°C (for 16S RNA) or 58°C (for *cmeR*). Melt-curve analysis was performed immediately following each amplification. Each specific amplicon was verified both by the presence of a single melting temperature peak and by the presence of a single band of expected size on a 3.5% agarose gel after electrophoresis. Control reactions with no RNA template were conducted for each primer set to ensure the absence of nonspecific-primer dimers. Samples were normalized using 16S RNA as an internal standard. Cycle threshold values were determined with the MyiQ software (Bio-Rad). The relative changes (*n*-fold) in *cmeR* transcription between the induced and noninduced samples were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (23).

RESULTS

Induction of *cmeABC* expression by antimicrobials. To determine if the expression of *cmeABC* can be conditionally induced by the substrates that are extruded by the efflux pump, we measured LacZ activities of strain JL110 (*C. jejuni* 81-176 containing P_{cmeABC}-*lacZ* [Table 1]) grown in the presence or absence of sublethal concentrations of antimicrobials. The LacZ assay showed that transcription of *cmeABC* was not affected by most of the tested substrates, including ethidium bromide, ciprofloxacin, norfloxacin, tetracycline, cefotaxime, rifampin, erythromycin, chloramphenicol, and salicylate (data not shown). However, addition of various bile salts to MH broth significantly induced the expression of *cmeABC* (Fig. 1). Compared to the basal level of transcription in MH broth, addition of various bile salts in the culture resulted in a 6- to 16-fold increase in the expression of *cmeABC* (Fig. 1A). The magnitudes of increase (*n*-fold) in LacZ activity upon induction by bile salts were reproducible in two to five independent experiments. Consistent with the increase in *cmeABC* transcription, immunoblotting showed that the production levels of CmeB and CmeC in cholate-containing media were substantially higher than those in the noninduced culture (Fig. 1B). Together, these results indicated that bile salts were strong inducers for the transcription and expression of *cmeABC*.

Induction of *cmeABC* by bile salts was dose dependent and time dependent. To determine the induction kinetics, the time

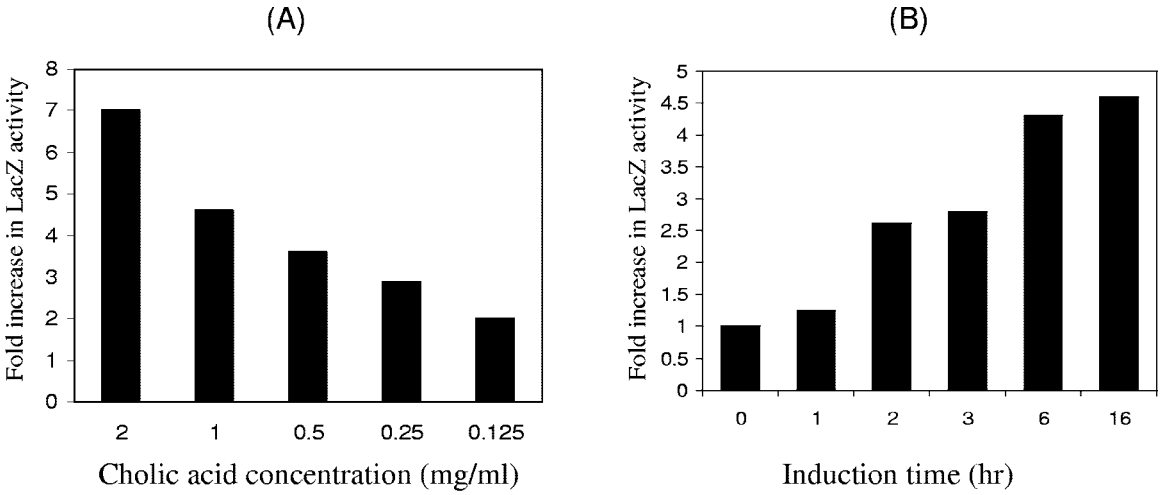

FIG. 2. Dose-dependent (A) and time-dependent (B) induction of the *cmeABC* operon by cholate. The data are presented as the ratios of LacZ activity between JL110 with cholate and the same strain grown without bile salts. Each bar represents the average LacZ ratio from triplicate samples.

course and dose response of the induction were measured using JL110 (Table 1). For measuring the dose-dependent response, JL110 was grown in MH broth containing different amounts of cholate for 16 h and then analyzed for LacZ activity. As shown in Fig. 2A, the sublethal concentration (2 mg/ml) of cholate led to an approximately sevenfold increase in LacZ activity over that of the noninduced culture. With a decrease of cholate concentration in each culture medium, the change in the transcription (*n*-fold) of *cmeABC* also declined (Fig. 2A). However, as little as 0.125 μg/ml of cholate in each culture medium still resulted in an approximately twofold induction in the transcription of *cmeABC* (Fig. 2A). The time course of the induction was determined using 1 mg/ml of cholate in MH broth, and the result is shown in Fig. 2B. A significant increase in transcription was observed after a 2-h incubation with cholate. Thereafter, the induction gradually increased and reached approximately fourfold that of the noninduced control after 6 h of growth. Further incubation up to 16 h yielded limited additional increase in transcription from that with the 6-h induction. Since work conducted with *E. coli* (42) and *Salmonella enterica* serovar Typhimurium (37) indicated that the bacterial growth phase affected the transcription level of *acrAB* (an RND-type efflux pump), we compared the transcription levels of *cmeABC* in the log phase and stationary phase by using JL110 in conventional MH broth, and the results from three independent experiments indicated that the transcription rate of *cmeABC* did not change with the growth phase (data not shown).

Bile salts interfere with CmeR binding to P_{cmeABC}. CmeR functions as a transcriptional repressor of *cmeABC* and binds specifically to P_{cmeABC}, inhibiting the expression of the efflux operon (19). We hypothesized that bile salts interact directly with CmeR, inhibiting the interaction between CmeR and P_{cmeABC} and leading to the increase in *cmeABC* expression. To test this hypothesis, we determined the interaction between CmeR and P_{cmeABC} in the presence or absence of bile salts by using SPR. Injection of the CmeR protein over a range of concentrations (13 to 208 nM) showed a steady-state increase in RU (data not shown), indicating the dose-dependent and stable binding of CmeR to the immobilized promoter DNA. This finding was consistent with the result obtained from the gel mobility shift assay conducted in a previous study (19) and further demonstrated the specific interaction between CmeR and P_{cmeABC}. As determined with BIA evaluation software, the dissociation constant (K_D) of CmeR to the promoter sequence was 88 ± 2 nM (χ^2 = 1.95).

SPR experiments were repeated using the same sensor chip and a constant concentration (104 nM) of CmeR, which was preincubated for 5 min with various amounts of sodium choleate (2 to 125 μg/ml) prior to injection. The results showed that when CmeR was exposed to increasing levels of sodium choleate, the binding of CmeR to the DNA decreased drastically (Fig. 3A). When sodium choleate was injected after CmeR bound to the immobilized P_{cmeABC} promoter DNA, a significant change in the dissociation rate of the CmeR-DNA complex was observed (Fig. 3B), indicating that bile salts promoted the dissociation of CmeR from the promoter DNA. This effect was proportional to the concentration of sodium choleate used (Fig. 3B). Together, the SPR results clearly demonstrated that bile salts interfere with the binding of CmeR to the promoter sequence of *cmeABC*, which is consistent with the finding that bile salts induce the expression of *cmeABC* in vivo (Fig. 1 and 2).

Bile salt does not affect the transcription of *cmeR*. To determine if bile salt affects the expression of *cmeR*, the transcription of *cmeR* in the presence of cholate (0.5, 1, and 2 mg/ml) was compared with that in the noninduced control by using real-time quantitative RT-PCR. Results from three independent experiments did not reveal any significant changes in the expression of *cmeR* in the presence of cholate (data not shown), indicating that the expression of *cmeR* was not induced or inhibited by bile salts. Thus, the enhanced expression of *cmeABC* in the presence of bile salts cannot be explained by the change in *cmeR* transcription.

CmeR-dependent and -independent induction. The results from the SRP experiments (Fig. 3) strongly suggest that the

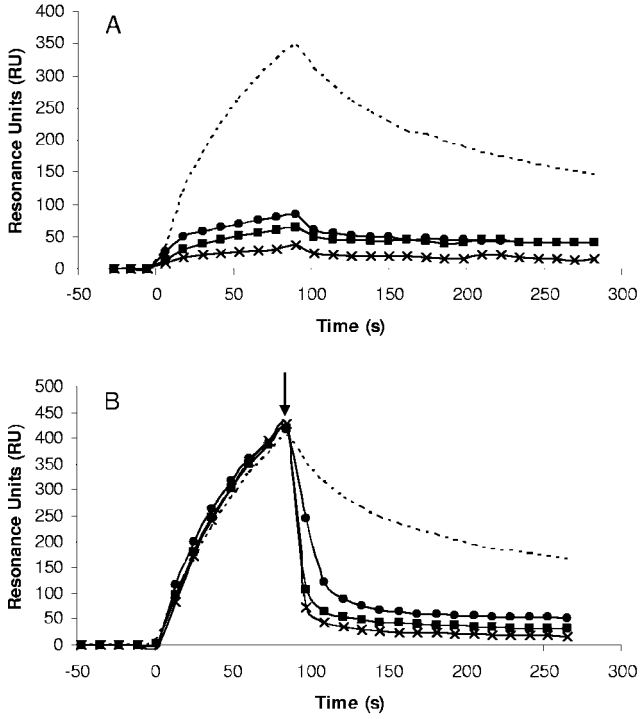

FIG. 3. SPR analysis of CmeR interaction with immobilized P_{cmeABC}. (A) CmeR (104 nM) was preincubated with sodium choleate at various concentrations, including 0 μg/ml (– –), 2 μg/ml (●), 16 μg/ml (■), and 125 μg/ml (×). The preincubated mixture (30 μl) was injected at a flow rate of 20 μl/min. (B) Sodium choleate at 0 μg/ml (– –), 2 μg/ml (●), 16 μg/ml (■), and 125 μg/ml (×) was injected just after the association phase by using the "co-inject" Biacore procedure. The arrow indicates the time of injection of sodium choleate. Each experiment was replicated at least three times, and all yielded similar results. This figure shows the results from one representative experiment.

induction of *cmeABC* by bile salts is mediated by release of the repression imposed by *cmeR* on *cmeABC*. To determine if the induction of *cmeABC* is fully CmeR dependent, the transcription of *cmeABC* was also measured in the *cmeR* null mutant (JL111) in the presence or absence of bile salts. Compared with that in JL110 (CmeR positive), the transcription of *cmeABC* in JL111 (CmeR negative) increased approximately fivefold in MH broth without bile salts (Fig. 4). In the presence of cholate, both JL110 and JL111 showed similar levels of increase (approximately fivefold) in the transcription of *cmeABC*, suggesting that the induction by cholate is fully CmeR dependent. However, the transcription level of *cmeABC* in JL110 in the presence of taurocholate exceeded that of the *cmeR* null mutant (JL111) and addition of taurocholate in the JL111 culture further enhanced the transcription of *cmeABC* over that in JL110 with taurocholate (Fig. 4). Since the *cmeR* null mutant displayed a fivefold increase in *cmeABC* transcription, the additional increase beyond the fivefold change in *cmeABC* transcription by taurocholate in JL110 and JL111 was not attributable to the release of CmeR-mediated suppression and suggests that a CmeR-independent pathway was also involved in the induction by taurocholate. Recently, Raphael et al. identified a *Campylobacter* response regulator (named CbrR) that is involved in bile resistance (40). To determine if

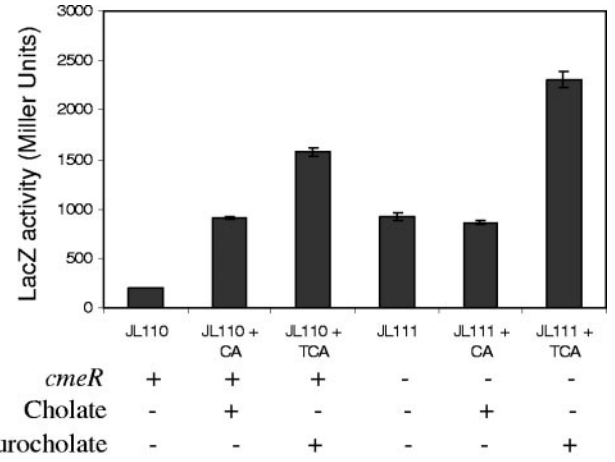

FIG. 4. CmeR-dependent and -independent induction of *cmeABC* by bile salts. JL110 and JL111 grown in MH broth only or with 1 mg/ml cholate (CA) or 12.5 mg/ml taurocholate (TCA) were used for the LacZ assay. The genetic backgrounds and the culture conditions are illustrated at the bottom. Each bar represents the mean LacZ activity ± standard deviation of triplicate samples.

CbrR was involved in the activation of *cmeABC* by bile salts, we introduced the *cbrR* mutation into JL110. When examined by the LacZ assay, the promoter activities of *cmeABC* were comparable between JL110 and JL112, regardless of the growth conditions (with or without cholate or taurocholate). This result suggests that CbrR is not involved in the activation of *cmeABC* in *C. jejuni*.

Bile salts increase resistance of *Campylobacter* to antibiotics. Since bile salts induced the expression of the CmeABC efflux system and CmeABC works synergistically with other mechanisms in conferring antimicrobial resistance (21, 25), we sought to determine if bile salts affected the susceptibility of *Campylobacter* to antibiotics. For this purpose, we compared the MICs of several antibiotics in strain 81-176 in the presence or absence of bile salts. As shown in Table 2, the presence of taurocholate in culture media resulted in a two- to fourfold increase in the resistance of *Campylobacter* to structurally divergent antibiotics, including cefotaxime, novobiocin, ciprofloxacin, fusidic acid, and erythromycin. The presence of cholate led to a twofold increase in MICs of some of the antibiotics but did not cause any measurable MIC changes with ciprofloxacin and erythromycin (Table 2). Consistent with the findings that taurocholate induced a higher level of transcrip-

TABLE 2. Effects of cholate (CA) and taurocholate (TCA) on the susceptibility of *C. jejuni* 81-176 to antibiotics

Antibiotic	MIC (μg/ml)[a]		
	No bile	With CA (1 mg/ml)	With TCA (25 mg/ml)
Cefotaxime	0.125	0.250 (2)	0.500 (4)
Novobiocin	16	32 (2)	64 (4)
Ciprofloxacin	0.0625	0.0625 (0)	0.125 (2)
Fusidic acid	64	128 (2)	256 (4)
Erythromycin	0.125	0.125 (0)	0.25 (2)

[a] The numbers in parentheses indicate the differences (*n*-fold) in MICs for strain 81-176 grown in the absence and presence of the corresponding bile salt.

tion of *cmeABC* than cholate did (Fig. 1 and 4), the MICs of the antibiotics assayed in the presence of taurocholate are reproducibly twofold higher than those in the presence of cholate (Table 2). Although the MIC changes were moderate, the results were reproducible in three independent experiments. Given the fact that bile conjugates are naturally present in animal intestinal tract, the results suggest that the presence of bile salts in vivo may decrease the susceptibility of *Campylobacter* to antibiotics.

DISCUSSION

Bacterial MDR pumps can be conditionally induced by their substrates, including antibiotics (6, 10, 11, 24, 27, 28, 47). Despite the fact that MDR pumps play important roles in antibiotic resistance, efflux of antibiotics by these pumps is considered an opportunistic function of the pumps, and the natural functions of the MDR efflux system are largely unknown (32, 35). Unlike antibiotics, natural antimicrobials such as bile salts are ubiquitously present in animal hosts and form a formidable barrier against invading bacteria in the intestinal tract. Thus, enteric bacteria have evolved multiple mechanisms for adaptation to the bile-containing habitat (12, 20). One of the major mechanisms involved in bile resistance is active efflux conferred by various membrane transporters, particularly the RND-type efflux pumps (22, 37, 48). In *Campylobacter*, inactivation of the *cmeB* gene resulted in a drastic increase in bile sensitivity and the *cmeB* null mutant was unable to colonize the intestinal tracts of chickens (22). In *E. coli*, *Salmonella*, and *Vibrio cholerae*, the AcrAB-TolC efflux pump (a homolog of the CmeABC pump) contributes to bile resistance and is inducible by bile salts (4, 37, 42). Given that bile acids are a natural component in the intestinal environment, the accumulating evidence on efflux-mediated bile resistance strongly suggests that efflux of bile acids is the natural function of some RND-type transporters (e.g., AcrAB-TolC and CmeABC) in enteric bacteria.

In this study, compelling evidence to demonstrate that bile salts modulate the production of CmeABC, a major efflux mechanism in *C. jejuni*, is provided. This conclusion is based on the facts that (i) transcription of *cmeABC* was greatly enhanced in the presence of various bile salts (Fig. 1 and 2), (ii) the production of the CmeABC proteins was increased in bile-containing media (Fig. 1), (iii) cholate inhibited CmeR binding to P_{cmeABC} (Fig. 3), releasing the repression on the transcription of *cmeABC*, and (iv) bile salts increased the resistance of *C. jejuni* to several antibiotics (Table 2), which agrees with the finding that the efflux machinery is elevated by bile salts. Since CmeABC is essential for bile resistance (22), the inducible expression of *cmeABC* by bile salts provides a flexible mechanism for *Campylobacter* to adapt in the intestinal environment. This notion is further supported by a recent study by Stintzi et al. (46), in which the expression of *cmeABC* was found to be highly up-regulated in rabbit ileal loops, as determined by whole-genome microarray. The elevated transcription of *cmeABC* in the rabbit ileal loops was likely the direct result of bile induction, although nonbile inducers for *cmeABC* may also exist in the gut.

Findings from this study revealed several unique features of CmeABC induction by bile salts in *C. jejuni*. First, expression of *cmeABC* can be significantly induced by both conjugated (e.g., taurocholate, glycocholate, and taurodeoxycholate) and unconjugated (e.g., cholate, deoxycholate, and chenodeoxycholate) bile salts. The major types of bile produced by the liver are the taurine and glycine conjugates of cholic acid and chenodeoxycholic acid, which are known as primary bile acids (48). These primary bile acids are secreted into the intestine, where deconjugation by residential microbes results in various secondary bile acid metabolites (e.g., deoxycholic acid and chenodeoxycholic acid). Thus, both conjugated and unconjugated bile salts exist in animal intestinal tract. In contrast to induction of CmeABC, induction of AcrAB in *E. coli* was obvious only with some unconjugated lipophilic bile salts (e.g., deoxycholate), while conjugated bile salts and other unconjugated bile salts (e.g., cholate) produced little or modest induction of AcrAB (42). In *S. enterica* serovar Typhimurium, transcription of *acrAB* was inducible by bile, but it is unclear which specific component of bile induces *acrAB* (37). However, deoxycholate was the only bile salt that activated transcription of *marRAB*, an operon involved in multidrug resistance in *E. coli* and *Salmonella* (37). Second, the magnitude of induction of *cmeABC* by bile salts in *C. jejuni* was much greater than that of *acrAB* in *E. coli*. Various bile salts at sublethal concentrations produced a 6- to 16-fold (Fig. 1A) induction of *cmeABC* (Fig. 1), while only up to a 1.7-fold induction was observed with *acrAB* in *E. coli* (42). Third, induction of *cmeABC* in *C. jejuni* is clearly dose dependent and time dependent (Fig. 2). Since CmeR is a cytoplasmic protein, intracellular accumulation of bile salts is required to achieve the induction of *cmeABC*, which may explain the time- and dose-dependent responses. For *E. coli* and *S. enterica* serovar Typhimurium, it is not clear if the induction of AcrAB by bile salts is kinetically similar to that of *cmeABC* in *Campylobacter*. Another difference is that growth phase affects the transcription of *acrAB* in *E. coli* and *S. enterica* serovar Typhimurium (37, 42), while growth phase had no effect on the transcription rate of *cmeABC* in *Campylobacter* (data not shown). Thus, the time-dependent manner of *cmeABC* induction by bile salts is unlikely an artifact caused by growth phase.

The CmeABC efflux system is regulated by CmeR, a TetR family repressor (19). The specific interaction between CmeR and P_{cmeABC} has been demonstrated in a previous study (19). Since the classical gel mobility shift assay is not always appropriate for assessing the interaction between inducers and regulators (31, 42), we chose to measure the effect of bile salts on CmeR binding to P_{cmeABC} in a real-time manner by using SPR. According to the data from the SPR experiments in this study, the K_D of CmeR to P_{cmeABC} is 88 ± 2 nM, which is in the same range as that reported for EthR (K_D = 146 nM), another repressor of the TetR/CamR family (8). By contrast, the affinities of MexL and CamR to their corresponding target promoters were low, with K_D values of 900 nM and 1,500 nM, respectively (1, 5). However, the K_D values of MexL and CamR were determined by gel mobility shift assays, instead of by SPR. According to the K_D values, the affinity of CmeR to P_{cmeABC} is substantially lower than that of TetR (K_D = 0.2 nM) to *tetO* (16). The difference in the K_D values is in agreement with previous findings showing that TetR tightly represses the *tetO* operator in the absence of tetracycline (33), while the *cmeABC* efflux pump is constitutively expressed at a moderate level in

spite of the repression by CmeR (19, 21, 34), suggesting a relatively loose control of *cmeABC* by CmeR.

The results from the SPR experiments (Fig. 3) also demonstrated that bile salts interfere with the binding of CmeR to P_{cmeABC} and promote the dissociation of CmeR from the immobilized P_{cmeABC}. This finding is consistent with the in vivo induction of *cmeABC* expression by bile salts (Fig. 1 and 2) and strongly suggests that bile-mediated inhibition of CmeR binding to P_{cmeABC} is responsible for the enhanced transcription of *cmeABC*. Transcriptional regulators of the TetR family are characterized by a conserved HTH-containing DNA-binding domain at the N-terminal region and a divergent C-terminal sequence that is involved in binding to various inducing ligands (11, 14, 15). Binding by an inducing compound to the C-terminal region triggers conformational changes in the N-terminal DNA-binding domain, reducing the affinity of a regulator to its target promoter DNA (10). Such structural changes in a repressor induced by the binding of a ligand have been confirmed for several regulator proteins in the TetR family, such as TetR and QacR (33, 44). Similar to other TetR family regulators, CmeR has a typical N-terminal DNA-binding HTH motif and a potential ligand-binding region in the C-terminal portion. Therefore, it is likely that bile salts interact with the C-terminal region of CmeR and induce conformational changes in the repressor, resulting in a great reduction in its DNA binding affinity. This speculation is in agreement with the results obtained from this study and needs to be confirmed by crystallization of the complex formed by CmeR and its inducing ligands. The real-time PCR result from this study indicated that transcription of *cmeR* was not affected by bile salts (data not shown), suggesting that the promoter activity of *cmeR* is not influenced by bile. Hence, altered expression of *cmeR* is not likely a factor in the induction of *cmeABC* by bile salts.

One of the interesting findings of this study is that both CmeR and a CmeR-independent pathway are involved in the induction of *cmeABC*. It appears that the induction by cholate is fully CmeR dependent, because both JL110 (with CmeR) and JL111 (without CmeR) showed similar levels of *cmeABC* transcription in the presence of cholate (Fig. 4). However, taurocholate further enhanced *cmeABC* transcription in the absence of a functional CmeR (Fig. 4), suggesting that a CmeR-independent pathway is also involved in the induction of *cmeABC* by taurocholate. It is possible that some bile salts (e.g., taurocholate) regulate another uncharacterized protein that subsequently activates the transcription of *cmeABC.* This hypothesis is conceivable because bile salts are known to influence the expression of multiple genes in bacterial cells and because multiple sensing mechanisms (including two-component systems) are involved in bacterial resistance to bile (3, 13, 20, 37, 43, 49). Recently, Raphael et al. (40) identified a response regulator (named CbrR) that is involved in bile resistance in *C. jejuni*. Inactivation of *cbrR* did not affect the induction of *cmeABC* by bile (data not shown), suggesting that CbrR is not involved in the activation of *cmeABC*. In *E. coli*, the major MDR efflux pump AcrAB is negatively regulated by the local repressor AcrR but positively regulated by global activators, including MarA, SoxS, and Rob (18). At this stage, it is unclear which regulator is involved in the CmeR-independent activation of *cmeABC* and how it modulates *cmeABC* expression in response to bile.

In summary, findings from this study revealed an induction-based mechanism that modulates the expression of *cmeABC* in response to bile salts, an environmental signal naturally occurring in the intestinal tract. This new finding plus previously identified roles of CmeABC in bile resistance and *Campylobacter* colonization highlight the significance of CmeABC in *Campylobacter* adaptation to the intestinal environment in animal hosts. Notably, in the presence of bile salts, *Campylobacter* showed increased resistance to several antibiotics due to overexpression of CmeABC (Table 2). Although the enhanced efflux itself cannot confer clinically relevant resistance to antibiotics, it may facilitate bacteria to better survive selection pressure and promote the emergence of antibiotic-resistant mutants via target gene mutations (e.g., *gyrA* mutations mediating fluoroquinolone resistance). This speculation is consistent with the observation that ciprofloxacin-resistant *Campylobacter* rapidly emerged in the intestinal tracts of chickens or humans treated with fluoroquinolone antimicrobials (25, 29, 45, 52). However, direct evidence is still lacking for bile-mediated enhancement of antimicrobial resistance in vivo. Thus, whether bile-induced expression of *cmeABC* influences the emergence of antibiotic-resistant *Campylobacter* in vivo remains to be examined in future studies.

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