CmeR Functions as a Transcriptional Repressor for the Multidrug Efflux Pump CmeABC in Campylobacter jejuni

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CmeR Functions as a Transcriptional Repressor for the Multidrug Efflux Pump CmeABC in Campylobacter jejuni

Abstract
CmeABC, a resistance-nodulation-division (RND) type of efflux pump, contributes to Campylobacter resistance to a broad spectrum of antimicrobial agents and is also essential for Campylobacter colonization of the animal intestinal tract by mediation of bile resistance. As one of the main systems for Campylobacter adaptation to different environments, CmeABC is likely subject to control by regulatory elements. We describe the identification of a transcriptional repressor for CmeABC. Insertional mutagenesis of cmeR, an open reading frame immediately upstream of the cmeABC operon, resulted in overexpression of cmeABC, as determined by transcriptional fusion (P<sub>cmeABC-lacZ</sub>) and immunoblotting with CmeABC-specific antibodies. Overexpression of the efflux pump was correlated with a moderate increase in the level of resistance of the cmeR mutant to several antimicrobials. In vitro, recombinant CmeR bound specifically to the promoter region of cmeABC, precisely, to the inverted repeat sequences in the cmeABC promoter. A single nucleotide deletion between the two half sites of the inverted repeat reduced the level of CmeR binding to the promoter sequence and resulted in overexpression of cmeABC. Together, these findings indicate that cmeR encodes a transcriptional repressor that directly interacts with the cmeABC promoter and modulates the expression of cmeABC. Mutation either in CmeR or in the inverted repeat impedes the repression and leads to enhanced production of the MDR efflux pump.

Disciplines
Genetics and Genomics | Molecular Genetics | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

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CmeR Functions as a Transcriptional Repressor for the Multidrug Efflux Pump CmeABC in Campylobacter jejuni

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CmeABC, a resistance-nodulation-division (RND) type of efflux pump, contributes to Campylobacter resistance to a broad spectrum of antimicrobial agents and is also essential for Campylobacter colonization of the animal intestinal tract by mediation of bile resistance. As one of the main systems for Campylobacter adaptation to different environments, CmeABC is likely subject to control by regulatory elements. We describe the identification of a transcriptional repressor for CmeABC. Insertional mutagenesis of cmeR, an open reading frame immediately upstream of the cmeABC operon, resulted in overexpression of cmeABC, as determined by transcriptional fusion (PcmeABC::lacZ) and immunoblotting with CmeABC-specific antibodies. Overexpression of the efflux pump was correlated with a moderate increase in the level of resistance of the cmeR mutant to several antimicrobials. In vitro, recombinant CmeR bound specifically to the promoter region of cmeABC, precisely, to the inverted repeat sequences in the cmeABC promoter. A single nucleotide deletion between the two half sites of the inverted repeat reduced the level of CmeR binding to the promoter sequence and resulted in overexpression of cmeABC. Together, these findings indicate that cmeR encodes a transcriptional repressor that directly interacts with the cmeABC promoter and modulates the expression of cmeABC. Mutation either in CmeR or in the inverted repeat impedes the repression and leads to enhanced production of the MDR efflux pump.

As a general and important mechanism for antimicrobial resistance, multidrug efflux systems (often named MDR pumps) contribute significantly to the intrinsic and acquired resistance to antibiotics in bacterial organisms (37, 40, 46). In addition to being key players in antibiotic resistance, MDR pumps also facilitate bacterial adaptation to deleterious environments where toxic compounds or metabolites are present. In bacteria, expression of MDR efflux pumps is usually controlled by transcriptional regulators that either repress or activate the transcription of the MDR efflux genes (14, 37, 40). Many of these regulators are local repressors that directly interact with the promoter regions of MDR efflux genes or operons. For example, repressors AcrR (Escherichia coli), QacR (Staphylococcus aureus), MtrR (Neisseria gonorrhoeae), and MexR (Pseudomonas aeruginosa) bind specifically to the promoter sequences of acrAB, qacA, mtrCDE, and mexAB, respectively, thereby inhibiting the expression of the corresponding MDR efflux gene(s) (9, 12, 17, 27). Mutations in the repressors or repressor-binding sequences impede the repression and result in overexpression of efflux pumps, which consequently increases bacterial resistance to structurally unrelated antimicrobial agents (9, 12, 16, 38, 42, 48). Recently, two-component systems were also found to be involved in the regulation of bacterial MDR pumps (3, 10, 33, 34). These examples illustrate the complexity and diversity of the regulatory mechanisms for bacterial MDR efflux pumps.

Campylobacter jejuni is the leading bacterial cause of human food-borne enteritis in many industrialized countries (11) and has become increasingly resistant to antimicrobials, compromising the effectiveness of antibiotic treatments (8, 44, 45, 53). One of the mechanisms used by Campylobacter for antimicrobial resistance is the CmeABC efflux system, a resistance-nodulation-division (RND) type of efflux pump recently identified in C. jejuni (24, 39). This efflux pump system consists of three members, including an outer membrane protein (CmeC), an inner membrane drug transporter (CmeB), and a periplasmic fusion protein (CmeA). These three proteins are encoded by a three-gene operon (cmeABC) and function together to form a membrane channel that extrudes toxic substrates directly out of Campylobacter cells (24). CmeABC contributes significantly to the intrinsic and acquired resistance of Campylobacter to structurally diverse antimicrobials (24, 26, 39). In addition, CmeABC plays a key role in bile resistance and is essential for Campylobacter growth in bile-containing media and colonization of the animal intestinal tract (25). These findings have defined the importance of CmeABC in the antimicrobial resistance and pathobiology of Campylobacter.

Even though basal production of CmeABC in wild-type strains occurs at a level that can readily be detected with antibodies specific to the efflux pumps (24), little is known about the regulatory mechanisms that modulate the expression of cmeABC in Campylobacter cells. Understanding the regulatory system for CmeABC will provide new insights into the mechanisms by which Campylobacter contributes to multidrug resistance (MDR) and adaptation to environmental changes. In this study, we report on the identification of CmeR as a transcriptional repressor for CmeABC. The cmeR gene is
CHLF and CHLR (Table 2) were used in the PCR with Pfu Turbo. GSR1 were used to amplify the 170-bp DNA fragment that contains the intercmeABCnM primers, 2.5 mM MgSO₄, and go of fermented with kanamycin (30 rpm in Luria-Bertani (LB) medium. When needed, LB media were supplemented with ciprofloxacin.

To insert the products were purified with a QIAquick PCR purification kit (Qiagen) and subsequently sequenced. To insert the cat gene cassette into the cmeR gene, primers

cmeA located immediately upstream of cmeA and encodes a 210-amino-acid (aa) protein that shares sequence and structural similarities to the members of the TetR family of transcriptional repressors. Using various approaches, we show that CmeR represses the transcription of cmeABC by directly binding to the promoter region (specifically, to the inverted repeat [IR]) of the efflux operon. Mutations in CmeR or the CmeR-binding site impede the repression and result in the overexpression of CmeABC and enhanced resistance to multiple antibiotics.

**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 Mueller-Hinton (MH) broth (Difco) or agar at 42°C under microaerobic conditions, which were generated with a CampyPak Plus (Becton Dickinson) gas pack in an enclosed jar. When needed, MH media were supplemented with kanamycin (30 μg/mL) or chloramphenicol (4 μg/mL). C. jejuni cells were grown at 37°C with shaking at 200 rpm in Luria-Bertani (LB) medium. When needed, LB media were supplemented with kanamycin (30 μg/mL) or ampicillin (100 μg/mL).

**PCR.** All primers used for PCR are listed in Table 2. PCR was performed in a volume of 100 μL containing 200 μM each deoxynucleoside triphosphate, 200 nM primers, 2.5 mM MgSO₄, 50 ng of Campylobacter genomic DNA, and 5 U of Taq DNA polymerase (Promega) or Pfu Turbo DNA polymerase (Stratagene). Cycling conditions varied according to the estimated annealing temperatures of the primers and the expected sizes of the products. To amplify the 0.9-kb coding sequence of cmeR from C. jejuni strain 81-176, primers F and R were designed from the genomic sequence of C. jejuni NCTC 11168 (35) and were used in the PCR along with the genomic DNA of strain 81-176 and Taq DNA polymerase. PCR products were purified with a QiAquick PCR purification kit (Qiagen) and subsequently sequenced. To insert the cat gene cassette into the cmeR gene, primers CHLF and CHLR (Table 2) were used in the PCR with Pfu Turbo DNA polymerase to amplify the entire cat gene from shuttle vector pUOA18 (49).

To determine the binding of CmeR to the cmeABC promoter, primers GSF and GSR1 were used to amplify the 170-bp DNA fragment that contains the intergenic region (IT) from wild-type strain 81-176 and its mutant, strain CR3e, for gel mobility shift assays. Reverse primers GSR2, GSR3, and GSR4 were used in conjunction with primer GSF to map the specific CmeR-binding site in the IT. The locations of these PCR primers are indicated in Fig. 1A.

**Sequence analysis and prediction of secondary structures.** PCR products were sequenced with an automated DNA sequencer (model 377; Applied Biosystems). Sequence analysis was performed with the Genetics Computer Group (GCG) Sequence Analysis Software Package (Oxford Molecular). The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) of the National Center for Biotechnology Information was used to search for homologous sequences and conserved domains in CmeR. The MOTIF program (http://motif.genome.jp) was also used to search for protein motifs in CmeR. The Peptidestructure program in GCG was used to search for protein motifs in CmeR. The MO addiction program in GCG was used to make an initial prediction of the secondary structures of CmeR. Other

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. jejuni</strong></td>
<td></td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>Wild type; genome sequence known</td>
</tr>
<tr>
<td>81-176</td>
<td>Wild type; isolated from a human</td>
</tr>
<tr>
<td>CR3e</td>
<td>81-176 derivative; spontaneous macrolide-resistant mutant obtained after stepwise selection with ciprofloxacin</td>
</tr>
<tr>
<td>9B6</td>
<td>81-176 derivative; cmeB::kan</td>
</tr>
<tr>
<td>JL106</td>
<td>81-176 derivative; cmeC::kan</td>
</tr>
<tr>
<td>JL107</td>
<td>81-176 derivative; cmeR::cm</td>
</tr>
<tr>
<td>JL108</td>
<td>81-176 containing pMW10</td>
</tr>
<tr>
<td>JL109</td>
<td>JL107 containing pMW10</td>
</tr>
<tr>
<td>JL110</td>
<td>81-176 containing pIT81</td>
</tr>
<tr>
<td>JL111</td>
<td>JL107 containing pIT81</td>
</tr>
<tr>
<td>JL112</td>
<td>81-176 containing pIT3c</td>
</tr>
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</table>

**E. coli**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thi hsdR17 (rK2-, mK2) relA1 supE44 [Δlac-proAB]F` traD36 proA8 lacF21ΔM15</td>
</tr>
<tr>
<td>DH5α</td>
<td>F- d88lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK2-, mK2) phoA supE44 thi-1 gyrA96 relA1 λ-</td>
</tr>
</tbody>
</table>

**TABLE 2. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>5'-TAGAAAAGATATATTGTTGATACCC-3'</td>
</tr>
<tr>
<td>R</td>
<td>5'-GGCCACTAACTTTGAGGTTTTA-3'</td>
</tr>
<tr>
<td>R1</td>
<td>5'-AATTTTTGGCTAATTATATT-3'</td>
</tr>
<tr>
<td>CHLF</td>
<td>5'-TGTCCTGCGCGGTTCCTTTT-3'</td>
</tr>
<tr>
<td>CHLR</td>
<td>5'-GGGCCCTTATGCTCTCAAAG-3'</td>
</tr>
<tr>
<td>RF</td>
<td>5'-ATAAGGATCCATGAACTCAAAGAAACAC-3' (BamHI)</td>
</tr>
<tr>
<td>RR</td>
<td>5'-TTTTAAGCTTTGGACATTTGATT-3' (HindIII)</td>
</tr>
<tr>
<td>GSF</td>
<td>5'-CTAAATGATGAACTTGAACCT-3'</td>
</tr>
<tr>
<td>GSR1</td>
<td>5'-GCACACACCTAAGCTCTAA-3'</td>
</tr>
<tr>
<td>GSR2</td>
<td>5'-TAAAAGTTGAAATTTGTTTACAG-3'</td>
</tr>
<tr>
<td>GSR3</td>
<td>5'-ATTGAAATTTTATGACGAAAT-3'</td>
</tr>
<tr>
<td>GSR4</td>
<td>5'-AAGCTTGGCTAATTGATT-3'</td>
</tr>
<tr>
<td>AR</td>
<td>5'-AATCCGCTGGTCATTTGCG-3'</td>
</tr>
<tr>
<td>PF</td>
<td>5'-AAAGGATCCTAAATGGAAATCGTTCTC-3' (BamHI)</td>
</tr>
</tbody>
</table>

¹ Underlining indicates restriction sites. The names of the restriction sites are listed in parentheses following the sequences.
and CHLR were used to amplify the 802-bp NCTC 11168 was transferred into strain 81-176 by natural transformation. The cat transformants by insertion of the containing 4 cmeR. The pCMERC construct, which served as a suicide vector, was electro-codon encoding aa 127 of CmeR in the same direction as the transcription of sequencing of the construct indicated that the directly ligated to BsrBRI-digested pCMER to obtain construct pCMERC. Se-

program, including SOMP and SOSUSI (BCM Search Launcher Texas, Baylor College of Medicine [http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html]), were also used to improve the prediction power.

Insertional mutation of cmeR. An isogenic cmeR mutant was first constructed by insertional mutagenesis in strain NCTC 11168. The cmeR mutation was then introduced into strain 81-176 by natural transformation. To construct the cmeR mutant in NCTC 11168, primers F and R1, designed from the published genomic sequence (35), were used to amplify a 729-bp fragment containing the entire open reading frame (ORF) of cmeR. The PCR product was cloned into the pGEMT-Easy vector (Promega), resulting in the construction of pCMER. Since a unique BsrBRI site (which generates blunt ends) occurs in the middle of cmeR, pCMER was digested with BsrBRI to interrupt the cmeR gene cassette from pUOA18 (49)

In vitro selection of fluoroquinolone-resistant mutants. Fluoroquinolone-susceptible wild-type strain 81-176 was used as the parental strain for plating. Spontaneous fluoroquinolone-resistant mutants were obtained by stepwise selection on MH agar plates containing ciprofloxacin (ICN Biomedicals Inc.). At the first step, 200 µl of 2-day cultures of 81-176 containing approximately 2 × 10^8 CFU were plated on MH agar plates supplemented with 4 µg of ciprofloxacin per ml. Resistant colonies were selected, one of which was used for further plating with an increased ciprofloxacin concentration. The selection and plating process was repeated three times, and the final concentration of ciprofloxacin used for plating was 80 µg/ml. One clone (clone CR3e) from the final selection step was chosen for use in this study.

Susceptibility tests. The MICs of different antimicrobials for Campylobacter were determined by a microtiter broth dilution method as described in a previous publication (24). Briefly, Campylobacter cultures were grown in MH broth to the late log phase and then diluted in MH broth to obtain an inoculum with approximately 2 × 10^7 CFU. The cultures were serially diluted twofold in 96-well microtiter plates with MH broth. The starting concentrations for the twofold dilution series were 100 mg/ml for cholic acid and cholate; 5 mg/ml for sodium dodecyl sulfate (SDS) and fusidic acid, 100 µg/ml for ciprofloxacin, norfloxacin, tetracycline, and cefotaxime; and 5 µg/ml for ampicillin, ethidium bromide, and erythromycin. The volume in each well was 120 µl. Two wells were used for each dilution of the antimicrobials. To each well, 5 µl of the bacterial inoculum was added, resulting in a final bacterial density of 8 × 10^9 CFU/ml. The microtiter plates were incubated for 2 days under mi-
crocrophiociples at 42°C. Three independent experiments were conducted to confirm the reproducibilities of the MIC patterns. The compounds used in these assays were purchased from Sigma Chemical Co. (norfloxacin, tetracycline, ampicillin, cefotaxime, erythromycin, fusidic acid, cholic acid, and cholate), ICN Biomedicals Inc. (ciprofloxacin), EM Science (SDS), and AMRESCO (ethidium bromide).

Production and purification of CmR. A full-length histidine-tagged recombinant CmeR (rCmR) was produced in E. coli by using the pQE-30 vector of the Qiagen Wizard kit. The complete coding sequence of cmeR in C. jejuni 81-176 was amplified with primers RF and RR (Table 2). A restriction site (underlined in the primer sequences in Table 2) was attached to the 5’ end of each primer to facilitate the directional cloning of the amplified PCR product into the pQE-30 vector. The amplified PCR product was digested with BamHI and HindIII and was then ligated into the pQE-30 vector, which had previously been digested with BamHI and HindIII. Cloning, expression, and purification of recombinant CmeR were performed by the procedures described previously (24, 54). The plasmid in the E. coli clone producing CmeR was sequenced, with no mutations in the coding sequence of cmeR detected.

Electrophoretic mobility shift assays. To determine the binding of CmeR to the operator region of cmeABC, electrophoretic mobility shift assays were performed by the procedure described by Alekshun et al. (2), with slight modifications. Primers GSF and GSR1 (Table 2) were used to amplify the 170-bp cmeR-cmeABC IT, which was then labeled at the 3’ end with digoxigenin-11-ddUTP (DIG-11-ddUTP) by using the DIG Oligonucleotide 3’-End Labeling kit (Roche Molecular Biochemicals). An internal cmeA fragment amplified with primers AF and AR (Table 2) was labeled with DIG-11-ddUTP and was used as the control DNA for the gel shift assay. The DIG-11-ddUTP-labeled DNA fragments (0.2 pmol) were incubated with purified CmeR in amounts ranging from 9.4 ng to 1.2 µg in 20 µl of binding buffer containing 20 mM HEPES (pH 7.6), 1 mM EDTA, 10 mM (NaH₂)SO₄, 5 mM dithiothreitol, 0.2% Tween 20, 30 mM KCl, and 25 ng of poly(dI-dC). The reaction mixtures were incubated at room temperature for 15 min and were then subjected to electrophoresis on a non-denaturing 6% (wt/vol) polyacrylamide gel in 0.25% TBE (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA [pH 8.0]) at 200 V for 45 min. The DNA in the gels was transferred to a nylon membrane with a vacuum blotter. DIG-labeled DNA was detected and visualized by using alkaline phosphatase-conjugated anti-DIG antibody and the chemiluminescent substrate CDP-Star (Roche Molecular Biochemicals). For the competition experiments, different amounts (50-, 150-, and 300-fold molar excesses) of unlabeled DNA were added as competitors during the binding assays. To identify the specific CmeR-binding site, reverse primers GSR2, GSRR3, and GSR4 (Table 2; Fig. 1A) were used with primer GSF to generate DNA fragments spanning different portions of the IT between cmeR and cmeA. The PCR fragments were labeled with DIG and used for the DNA-binding assays, as described above.

Construction of promoter fusions. A 640-bp DNA fragment containing P cm::cmeR and its flanking region was amplified from wild-type strain 81-176 with primer pair PF and PR (Table 2; Fig. 1A). The amplified PCR products were digested with BamHI and XbaI and then inserted into plasmid pMW10, a shuttle vector carrying a promoterless lacZ gene (52), to create plasmid pIT81. The same promoter region in mutant CR3e was also amplified by PCR and inserted into pMW10 to create plasmid pIT3e. Plasmids pIT81, pIT3e, and pMW10 were mobilized into various C. jejuni strains by triparental mating using DH5α/pRK2013 (6) as the helper strain, according to the procedure described by Miller et al. (31).

β-Galactosidase assay. The β-galactosidase (LacZ) activity in the Campylobacter strains containing the P cm::cmeABC-lacZ transcriptional fusion was measured as described by Miller (30), with the modification that C. jejuni cultures were grown for 16 h in MH broth to log phase (absorbance at 600 nm, approximately 0.2) before they were harvested. All assays were conducted in triplicate.

Nucleotide sequence accession number. The cmeR gene sequence of C. jejuni 81-176 determined in this study was deposited in GenBank under accession AF466820.

RESULTS

Sequence features of cmeR and IT between cmeR and cmeABC in C. jejuni 81-176. Analysis of the genomic sequence of C. jejuni NCTC 11168 (35) suggested that Cj0368c, an ORF immediately upstream of the cmeABC operon, likely encodes a transcription factor. The homolog of Cj0368c in strain 81-176 was amplified by PCR with primers P and R (Fig. 1A; Table 2) and subsequently sequenced and was named cmeR in this study. CmeR encodes a 210-aa protein and is transcribed in the same direction as cmeABC (Fig. 1A). The deduced amino acid sequence of CmeR in strain 81-176 is 99.5% identical to the encoded product of Cj0368c in strain NCTC 11168. CmeR shares sequence similarities with the members of TetR family of transcriptional repressors of efflux systems (P4am accession number PF00440). In particular, the N-terminal region of CmeR contains a DNA-binding domain that is highly conserved among the TetR family regulators (Fig. 1B), including QacR (GenBank accession number AF053772; 50% identity in 52 aa of overlap) of S. aureus, ActR (GenBank accession number U00734; 40% identity in 60 aa of overlap) of E. coli, and MtrR (GenBank accession number Z25797; 31% identity in 83 aa of overlap) of Neisseria gonorrhoeae. Within the domain, an α-heelix–turn–α-helix (HTH) DNA-binding motif, a signature sequence of the TetR family regulators, is also present in CmeR (aa 30 to 60) (Fig. 1B). There is a 97-bp IT between cmeR and cmeA, in which an IR consisting of 7-bp half sites separated by a 2-bp spacer was identified (Fig. 1A). On the basis of the reported consensus promoter sequence of Campylobacter (52), the putative −10, −16, and −35 sequences were identified for the promoter of cmeABC in C. jejuni 81-176 (Fig. 1A). The IR is located between the predicted −10 and −35 sequences, and the −35 region partly overlaps with the half site of the IR. These sequence features suggested that CmeABC is subject to regulation and CmeR is likely a local regulator for CmeABC.

Insertional mutagenesis of cmeR increases the level of cmeABC expression. To determine if CmeR functions as a regulator for CmeABC, CmeR was inactivated by inserting the chloramphenicol resistance gene (cat) cassette into the codon encoding aa 127 of CmeR (Fig. 1A). As shown by immunoblotting (Fig. 1C, lane 4), the level of production of CmeB in the cmeR mutant (named JL107) of strain 81-176 was substantially higher than that in the wild type. Spot densitometric analysis of the immunoblot estimated that JL107 produced approximately fivefold more CmeB than wild-type 81-176. To determine if the increased level of production of CmeB in JL107 was due to an elevated level of transcription of cmeABC, the promoter sequence (P cm::cmeABC) of cmeABC in 81-176 was placed upstream of the promoterless lacZ gene in plasmid pMW10 to create transcriptional fusion plasmid pIT81 (Table 1), which was then transformed into wild-type strain 81-176 and JL107. As shown in Table 3, the LacZ activity in JL108 and JL109 (which carried the control plasmid pMW10) was barely detectable, indicating that the endogenous level of expression of the promoterless lacZ was low and negligible. On the basis of the measurement of β-galactosidase activity, P cm::cmeABC was moderately active (236 Miller units) in wild-type strain 81-176.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>β-Galactosidase activity (Miller units)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>JL108</td>
<td>81-176 with pMW10</td>
<td>1.45 ± 0.25</td>
</tr>
<tr>
<td>JL109</td>
<td>JL107 (cmeR::cm) with pMW10</td>
<td>2.05 ± 0.30</td>
</tr>
<tr>
<td>JL110</td>
<td>81-176 with pIT81</td>
<td>2.36 ± 2</td>
</tr>
<tr>
<td>JL111</td>
<td>JL107 (cmeR::cm) with pIT81</td>
<td>1.467 ± 21</td>
</tr>
<tr>
<td>JL112</td>
<td>81-176 with pIT3e</td>
<td>586 ± 20</td>
</tr>
</tbody>
</table>

* Means of triplicate measurements ± standard deviation.
(shown for strain JL110 in Table 3), which was consistent with the fact that the CmeABC proteins were detectable by immunoblotting in the wild-type strain (Fig. 1C) (24). However, in the absence of a functional CmeR, the level of transcription of P_{cmeABC} was elevated approximately 6.2-fold (to 1,467 Miller units; shown in strain JL111) over the wild-type level. The fold difference in the level of transcription of P_{cmeABC} between wild-type strain 81-176 and the cmeR mutant was comparable to that from the immunoblotting results, shown in Fig. 1C. Inactivation of CmeR in strain NCTC 11168 also resulted in cmeABC overexpression (data not shown), further confirming the role of CmeR in controlling cmeABC. Together, these results indicate that CmeR represses the transcription of cmeABC and that inactivation of CmeR results in the overexpression of the efflux operon.

**CmeR binds to the IT between cmeR and cmeA.** To determine if CmeR regulates the cmeABC operon via direct interaction with the promoter of cmeABC, a gel mobility shift assay was performed with rCmeR and the IT DNA amplified with primers GSF and GSR1 (Fig. 1A; Table 2). rCmeR showed a molecular mass of approximately 23 kDa on SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2A), consistent with the calculated molecular mass from the deduced amino acid sequence of CmeR. As shown in Fig. 2B, rCmeR bound to the IT DNA but not to the control DNA, which was a 170-bp internal fragment of cmeA amplified by PCR with primers AF and AR (Table 2). The specific interaction between rCmeR and the IT DNA was further confirmed by a competition assay (Fig. 2C). The addition of a 50-fold molar excess of the unlabeled IT DNA (Fig. 2C, lane 3) completely eliminated the formation of the DNA-protein (D-P) complex, while the 170-bp control DNA did not have any effect on rCmeR binding to the IT, even at a molar excess as high as 300-fold (Fig. 2C, lanes 6 to 8). Together, these findings indicate that CmeR specifically binds to the promoter region of cmeABC. When different concentrations of rCmeR were used in the DNA-binding assay, a single retarded D-P complex was always observed on the gel (data not shown), suggesting that there is only one CmeR-binding site in the IT region.

**The IR in the IT is required for specific CmeR binding.** Regulators of bacterial MDR efflux systems usually bind to the IRs in their target promoter regions (14). The presence of an IR upstream of cmeABC (Fig. 1A) suggested a potential binding site for CmeR. To examine this possibility, the gel mobility shift assay was performed with a series of PCR products whose sequences span different portions of the IT region between cmeR and cmeABC (see Fig. 1A for the locations of the primers). As shown in Fig. 3A, rCmeR bound to the DNA fragments amplified by primers GSF and GSR1 or primers GSF and GSR2, and partial binding was also observed with the fragment amplified with primers GSF and GSR3. However, the PCR fragment lacking the IR derived with primers GSF and GSR4 was not bound by rCmeR, because no retarded rCmeR-DNA complex was observed on the gel (Fig. 3A, lane 8). These observations strongly indicate that the IR is the binding site for CmeR. The reduced level of binding of CmeR to the fragment obtained by PCR with primers GSF and GSR3 suggested that the immediate flanking sequence of the IR is also required for full binding by CmeR.

**Mutation in the IR affects expression of cmeABC.** A spontaneous MDR mutant (designated CR3e) of strain 81-176, obtained by stepwise selection on ciprofloxacin-containing plates, showed high levels of resistance to fluoroquinolones and concurrently elevated levels of resistance to several other antibiotics (Table 4), although ciprofloxacin was the exclusive antimicrobial used in the stepwise selection. Immunoblotting analysis of the CmeABC proteins in CR3e indicated that the efflux proteins are overexpressed in this mutant (Fig. 1C, lane 5). To determine the mechanism responsible for the increased level of production of CmeABC in CR3e, the complete cmeR gene and the IT region between cmeR and cmeA were ampli-
tion of cmeABC, the P\textsubscript{cmeABC} sequence bearing the single deletion mutation was transcriptionally fused to the promoter-less lacZ in pMW10 to create pIT3e, which was then introduced into wild-type strain 81-176 to create strain JL112 (Table 1). As shown in Table 3, the β-galactosidase activity was approximately 2.5-fold higher in JL112 (which contained the mutated promoter-lacZ fusion) than in JL110 (which contained the wild-type promoter-lacZ fusion), indicating that the mutated P\textsubscript{cmeABC} is more active than the wild-type P\textsubscript{cmeABC} and that the single deletion mutation increases the level of transcription of cmeABC in CR3e.

**Overexpression of cmeABC correlates with enhanced resistance to multiple antibiotics.** Compared to wild-type strain 81-176, the CmeR mutant (strain JL107) showed enhanced resistance to several antibiotics (Table 4). The MICs of ciprofloxacin, norfloxacin, cefotaxime, and fusidic acid for JL107 increased twofold and the MIC of erythromycin for JL107 increased fourfold. The MIC results and the moderate differences in the MICs for the cmeR mutant and those for wild-type strain 81-176 were reproducible in three independent experiments. Seven consecutive passages (~168 generations) of JL107 in MH broth showed the same MIC changes, indicating that the phenotype is stable. Although tetracycline, ampicillin, ethidium bromide, cholic acid, choleate, and SDS are substrates of CmeABC (24), mutant JL107 showed only a wild-type level of resistance to these substances. Overexpression of cmeABC in spontaneous mutant CR3e also appeared to be correlated with the MDR phenotype of the mutant. Although the Thr-86-Ile point mutation was present in the gyrA gene of CR3e (data not shown), the exceedingly high level of resistance to ciprofloxacin (Table 4) may be explained by the overexpression of cmeABC. In addition, the enhanced resistance of CR3e to tetracycline, ampicillin, cefotaxime, erythromycin, and fusidic acid was at least partially attributable to the overexpressed cmeABC.

**DISCUSSION**

This work demonstrates that CmeR functions as a transcriptional repressor for CmeABC and that the interaction of CmeR with the IR immediately upstream of cmeABC regulates the expression of this MDR operon. This conclusion is based

**TABLE 4. Susceptibilities of C. jejuni 81-176, JL107, and CR3e to different antimicrobials**

| Antimicrobial     | MIC [µg/ml]
|-------------------|------------
|                   | 81-176 | JL107 | CR3e |
| Ciprofloxacin     | 0.390 | 0.780 (2) | 100 (256) |
| Norfloxacin       | 0.098 | 0.196 (2) | 6.25 (64)  |
| Tetracycline      | 0.098 | 0.098 (--) | 0.196 (2)  |
| Ampicillin        | 0.625 | 0.625 (--) | 2.5 (4)     |
| Cefotaxime        | 0.390 | 0.780 (2) | 1.56 (4)    |
| Erythromycin      | 0.039 | 0.156 (4) | 0.156 (4)   |
| Ethidium bromide  | 0.625 | 0.625 (--) | 0.625 (--)  |
| Fusidic acid      | 39    | 78 (2)    | 78 (2)      |
| Cholic acid       | 3,125 | 3,125 (--) | 3,125 (--)  |
| Choleate          | 12,500| 12,500 (--) | 25,000 (2) |
| SDS               | 156   | 156 (--)  | 156 (--)    |

*The numbers in parentheses indicate the fold differences in MICs between 81-176 and its mutant derivatives. --, no MIC difference was observed.*
on the following evidence. First, CmeR shares significant sequence and structural homologies with known repressors belonging to the TetR family of transcriptional regulators. Second, inactivation of CmeR by insertional mutagenesis substantially increased the level of transcription of cmeABC and, consequently, enhanced the level of production of the CmeABC proteins (Table 3 and Fig. 1C). Third, CmeR specifically bound to the unique IR upstream of cmeA, as shown by the gel mobility shift assay (Fig. 2 and Fig. 3A). Finally, a mutation in the IR (a 1-bp deletion between the two half sites) significantly reduced the level of binding by CmeR (Fig. 3B) and resulted in enhanced transcription (Table 3) and translation (Fig. 1C) of the efflux operon. Together, these findings formally define the critical role of CmeR and its specific binding site on the regulation of CmeABC in C. jejuni.

CmeR represses the transcription of cmeABC, but it also allows a moderate level of production of the efflux proteins in wild-type strains in the absence of antibiotics. This feature is different from the control of TetA by TetR, in which the basal level of expression of tetA is minimal in the absence of tetracycline (14). The difference is probably due to the fact that the TetA pump is specific for tetracycline and constitutive expression of tetA is not required in the absence of the substrate (19). In contrast, the other efflux pumps that are controlled by repressors of the TetR family and that have a broad spectrum of substrates (e.g., AcrAB, MtrCDE, and QacA) are expressed at substantial levels in wild-type strains even in the absence of specific substrates (12, 15, 48). This relatively high basal level of expression of the MDR pumps is probably required for their key roles in conferring intrinsic resistance to different antimicrobials and toxic compounds and facilitates the adaptation of bacterial organisms to environmental changes. In addition, bacterial MDR pumps are likely required for extrusion of endogenous toxic metabolites (18, 37), which also necessitates constitutive expression of the efflux pumps even in the absence of exogenous selection pressure. On the other hand, the overproduction of efflux pumps in the absence of selection pressure or substrates has been demonstrated to be deleterious to some organisms (7, 32, 43). Therefore, there is a need for regulatory systems to modulate the expression of MDR efflux pumps in bacteria. In this respect, CmeR acts as a modulator in Campylobacter to maintain balanced production of CmeABC to meet the physiological needs and facilitate the adaptation of Campylobacter to environmental changes, including antibiotic treatments.

The cmeR gene was inactivated by the insertion of an antibiotic resistance gene cassette in the middle of the ORF (Fig. 1). Although there was a possibility that the truncated N-terminal portion of CmeR was still produced in the mutant strain, it is unlikely that the truncated version of CmeR was functional in repressing the transcription of cmeABC. CmeR belongs to the TetR family, and the members of this family function as dimers. Although the DNA-binding motif is located in the N-terminal portion, the C-terminal portion is essential for dimer formation (14). Deletion of the C-terminal portion of the regulatory protein in the TetR family would prevent the formation of dimers and, consequently, would affect the binding to target DNA. Hence, the truncated CmeR, even if it were produced in the mutant strain, is not expected to perform the repressor function, as is the case with the full-length CmeR. This argument is directly supported by the findings that the CmeR mutant showed a significant increase in the level of transcription of cmeABC and the level of production of the efflux proteins (Table 3 and Fig. 1). Regardless of the expression status of the truncated CmeR in the mutant, the results from this study demonstrate that CmeR functions as a repressor for CmeABC. To determine if CmeR directly bound to the promoter sequence of cmeABC, His-tagged rCmeR was produced in E. coli and was used for the gel mobility shift assay. His-tagged recombinant proteins have commonly been used to assess the binding of MDR pump repressors to target DNA (9, 12, 42). It was unlikely that the His tag attached to rCmeR had any effect on DNA binding because rCmeR did not bind to the negative control DNA (internal cmeA fragment), while it bound specifically to the promoter DNA of cmeABC (Fig. 2). In addition, the binding specificity was further verified by competition with the nonlabeled promoter DNA and mutation of the binding site (Fig. 2 and 3).

The IR is a typical DNA motif for binding sites of regulatory proteins (14). It has been known that the correct spacing between the two half sites of an IR is critical for binding by repressors (13, 51). For example, Wissmann et al. (51) showed that a 1-bp increase or decrease in the single-base-pair spacing between the two half sites of the tet operator decreased the affinity of the operator sequence to TetR. Another study with staphylococcal QacR (13) also demonstrated that binding of QacR was dependent on correctly spaced operator half sites. In this study, we found that CmeR bound specifically to the IR in the promoter region of CmeABC (Fig. 3) and that a single nucleotide deletion between the two half sites of the IR reduced the level of CmeR binding to the promoter sequence of cmeABC (Fig. 3B) and led to a 2.5-fold increase in the level of transcription of P_cmeABC (Table 3). On the basis of these findings, we can confidently link the enhanced expression of cmeABC in mutant CR3 to the single nucleotide deletion in the IR. The sequence feature of the IR upstream of cmeABC is similar to that of the IR bound by TetR, which comprises 9-bp half sites separated by a 1-bp spacer (14, 51), but is different from that of the large QacR-binding region comprising 15-bp half sites separated by a 6-bp spacer (12). On the basis of the sequence analogy of the binding sites and the known binding mechanisms of TetR and QacR (13, 20), it is speculated that CmeR may bind to its operator as a dimer in a way similar to that of TetR rather than to a pair of dimers, as is the case with QacR (13). This speculation remains to be examined in future studies.

Overexpression of MDR efflux pumps mediated by mutations in their regulatory elements is usually associated with acquired resistance to multiple antibiotics in bacteria (36, 37, 50). The results from this study also indicate that overexpression of CmeABC increases the levels of resistance of Campylobacter to several antimicrobials (Table 4). On the basis of the MIC, the enhanced resistance in the isogenic CmeR mutant (strain JL107) was moderate, but the differences were reproducible in independent experiments. At this stage it is unclear why the overexpression of CmeABC mediated by the CmeR mutation did not cause large changes in the MICs for the CmeR mutant. There is a possibility that CmeR also regulates other unidentified genes and that inactivation of CmeR may have pleiotropic effects on gene expression in Campylobacter.
which potentially obscures the changes in MICs. This possibility is being examined in our laboratory. Nevertheless, the relatively small-scale increase in the resistance profiles of the CmeR mutant is not totally surprising, because it has been found in other bacteria that overexpression of a single MDR pump caused by mutations in its local repressor may not confer drug resistance to a level of clinical significance (21, 38, 47). However, overexpression of MDR pumps may allow bacteria to survive under the pressure of high antibiotic concentrations and promote the emergence of mutants with specific target gene mutations that are highly resistant to antimicrobials (46, 50). The contribution of CmeABC overexpression to the acquired antibiotic resistance in Campylobacter remains to be determined in future studies.

In mutant CR3e, which was obtained by stepwise selection on ciprofloxacin-containing plates, the high-level resistance to fluoroquinolones (Table 4) was expected because the mutant contained the specific GyrA mutation (Thr-86-Ile), which, in conjunction with the function of CmeABC, confers a high level of resistance to fluoroquinolones (26). The enhanced resistance of CR3e to other antimicrobials (Table 4) is at least partially attributable to the overexpression of CmeABC in the mutant. However, there is a possibility that other unknown mutations might also have occurred, and these might also have contributed to the increased level of antibiotic resistance in CR3e. Due to technical difficulties, our effort to introduce the single nucleotide deletion in the cmeABC promoter of CR3e into wild-type 81-176 by using natural transformation or electroporation was not successful. Thus, the involvement of unknown mutations in the enhanced resistance in CR3e cannot be totally excluded at this stage.

The expression of MDR efflux pumps can be conditionally induced by structurally diverse substrates of these pumps (1, 5, 12, 22, 23, 28, 29, 41). This induction is due to the direct interaction of the substrates with repressor molecules, which interfere with the binding of repressors to operator DNA and which results in increased levels of expression of MDR pump genes. 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 the binding to inducing compounds (12, 19, 20). The variation in the C-terminal sequence reflects the diversity of substrates that can interact with the regulators. A conformational change occurs in the DNA-binding domain when an inducer binds to the C-terminal region of a repressor, releasing the inhibition to efflux pumps. Although CmeABC is the key pump for bile resistance in Campylobacter (25), no differences in the MICs of cholic acid and cholate were observed for the wild-type strain 81-176 and strain JL107 (an isogenic cmeR mutant of strain 81-176) (Table 4). Considering the fact that bile salts induce AcrAB expression in E. coli (41), it is possible that bile salts also induce cmeABC expression in Campylobacter. If this induction indeed occurs, it would obscure the differences in the MICs of bile salts for wild-type strain 81-176 and the CmeR mutant due to the enhanced expression of cmeABC in strain 81-176 in the presence of bile salts. At this stage, it is unclear if the expression of cmeABC is inducible and if any substrates directly interact with CmeR. Since we have constructed the transcriptional reporter system (P_{cmeABC-lacZ}) and established the gel mobility shift assay using rCmeR, the induction of cmeABC under various conditions can now be examined in a definitive manner.

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