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The *Campylobacter jejuni* Response Regulator, CbrR, Modulates Sodium Deoxycholate Resistance and Chicken Colonization

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Two-component regulatory systems play a major role in the physiological response of bacteria to environmental stimuli. Such systems are composed of a sensor histidine kinase and a response regulator whose ultimate function is to affect the expression of target genes. Response regulator mutants of *Campylobacter jejuni* strain F38011 were screened for sensitivity to sodium deoxycholate. A mutation in Cj0643, which encodes a response regulator with no obvious cognate histidine kinase, resulted in an absence of growth on plates containing a subinhibitory concentration of sodium deoxcholate (1%, wt/vol). In broth cultures containing 0.05% (wt/vol) sodium deoxycholate, growth of the mutant was significantly inhibited compared to growth of the *C. jejuni* F38011 wild-type strain. Complementation of the *C. jejuni cbrR* mutant in *trans* restored growth in both broth and plate cultures supplemented with sodium deoxycholate. Based on the phenotype displayed by its mutation, we designated the gene corresponding to Cj0643 as *cbrR* (*Campylobacter bile resistance regulator*). While the MICs of a variety of bile salts and other detergents for the *C. jejuni cbrR* mutant were lower, no difference was noted in its sensitivity to antibiotics or osmolarity. Finally, chicken colonization studies demonstrated that the *C. jejuni cbrR* mutant had a reduced ability to colonize compared to the wild-type strain. These data support previous findings that bile resistance contributes to colonization of chickens and establish that the response regulator, CbrR, modulates resistance to bile salts in *C. jejuni*.

Campylobacter jejuni is a leading reported cause of bacterial gastroenteritis worldwide. Campylobacteriosis is characterized by diarrhea containing blood and leukocytes. The pathogenesis of campylobacteriosis involves the destruction of colonic epithelium and invasion of the lamina propria by the bacterium. The infectious dose of *C. jejuni* is variable but has been reported to be as low as 800 organisms (9). Thus, *C. jejuni* must be able to resist a variety of antimicrobial factors found in the digestive system of its host, including pH variations, digestive enzymes, and bile.

Bile is secreted by the liver and is composed primarily of bile salts and to a lesser extent phospholipids and cholesterol (12). The primary bile salts are cholic acid, glycocholic acid, deoxycholic acid, and taurocholic acid, and deoxycholic acid constitutes approximately 15% of the total (16). The average concentration of bile salts in the human intestine ranges from 0.2 to 2% (16). In chickens, a natural host for *C. jejuni*, the concentration of bile salts ranges from 0.01% in the cecum to 0.7% in the jejunum (28). While bile salts aid the host in the digestion of fats, they also serve as an effective antimicrobial agent by disaggregating the lipid bilayer of cellular membranes (19). In gram-negative bacteria, bile salts can pass directly across the outer membrane or pass through porins (e.g., OmpF in *Escherichia coli*) (36). Thus, enteric pathogens have developed mechanisms to resist the damaging effects of bile salts.

In *C. jejuni*, the only characterized mechanism of bile salt resistance is active efflux as a function of the multidrug efflux pump CmeABC (27, 28). A *C. jejuni cmeB* mutant shows increased sensitivity to antimicrobial agents, including different classes of antibiotics, detergents, and heavy metal-containing salts. The *C. jejuni cmeB* mutant is 64-fold more sensitive to sodium deoxycholate than a wild-type strain and is unable to colonize the intestines of experimentally inoculated chickens (28).

One mechanism that bacteria use to sense and respond to the presence of bile (salts) includes a two-component regulatory (TCR) system (19). The TCR paradigm involves two components, a histidine/sensor kinase (HK) and a cytoplasmic protein termed the response regulator (RR) (4, 24). A typical HK consists of two functional domains, an N-terminal signal or input domain and a C-terminal sensor kinase. The N-terminal domain may directly interact either with a signal or with another protein that serves to relay the signal to the input domain. In cases where the signal is external, the HK is a membrane-spanning protein with a periplasmic input domain. Upon receiving the stimulus, the signal domain activates the C-terminal sensor kinase domain. The activated sensor kinase hydrolyzes ATP, causing the autophosphorylation of a conserved histidine residue in the sensor kinase domain. Upon HK phosphorylation, interaction with the RR N-terminal receiver domain allows transfer of the phosphate, via a phosphorelay system, to a conserved aspartic acid residue. This phosphorylation activates the C-terminal output domain of the RR, which often results in the binding of the RR to DNA via a DNA-binding motif. This DNA binding may induce or repress genes controlled by the TCR system. Some RRs lack a DNA-

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binding domain; however, these RRs may bind other proteins, or they may transfer a phosphate to another HK or RR.

Analysis of the *C. jejuni* genome has identified six HKs, 11 RRs, and an HK-RR hybrid (CheA) (31). Four of the genes encoding *C. jejuni* RRs, *cheY*, *flgR*, *racR*, and *dccR*, have been characterized to date (10, 22, 26, 29, 40, 42). Yao et al. (42) reported that a *C. jejuni cheY* null mutant was nonmotile when it was grown in semisolid media and that it was attenuated in a ferret model, suggesting that chemotaxis is important in establishing colonization and/or pathology in the host environment. Hendrixson and DiRita (23) showed that a *cheY* mutation affected colonization of chickens. The *racR* gene of *C. jejuni* was identified as the RR component associated with a temperature-dependent signaling pathway in *C. jejuni* (10). A *C. jejuni racR* null mutant displayed temperature-dependent changes in its protein profile and growth characteristics, and its ability to colonize the intestines of chickens was reduced (10). Jagannathan et al. (26) and Hendrixson and DiRita (22) showed that mutations in FlgR affected motility. More recently, Wosten et al. (40) found that a TCR system, FlgS/FlgR, was involved in the gene regulation of the flagellar apparatus. Specifically, phosphorylated FlgR activates transcription of σ^{54}-dependent genes that encode the hook and basal body structures of the flagellum. Finally, the DccRS system appears to be required for colonization of chickens and mice; however, it is not clear what the functions of the DccRS-controlled genes are (29).

In this study, we sought to identify TCR systems involved in the resistance of *C. jejuni* to deoxycholate. Mutations were generated in 9 of 11 genes encoding RRs, most of which were predicted to interact directly with the promoters of specific target genes via a DNA-binding domain. We chose to mutate RRs exclusively since HKs may interact with more than one RR. Here we show that a *C. jejuni cbrR* mutant is unable to survive in the presence of bile salts and other detergents and poorly colonizes experimentally inoculated chickens.

MATERIALS AND METHODS

Bacterial strains. *C. jejuni* strain F38011 was grown on Mueller-Hinton (MH) agar plates containing 5% citrated bovine blood (MH-blood) in a microaerobic atmosphere. Where indicated below, MH-blood plates were supplemented with either chloramphenicol (15 μg/ml) or kanamycin (200 μg/ml). *E. coli* InvαF′ was grown either in Luria-Bertani broth or on Luria-Bertani agar plates. When appropriate, the plates were supplemented with chloramphenicol (15 μg/ml) or kanamycin (50 μg/ml).

Mutagenesis of RRs. For each RR, oligonucleotide primers (Table 1) were used to amplify the upstream and downstream regions of the target gene such that approximately 100 bp of the open reading frame was omitted. More specifically, an approximately 600-bp fragment including the 5′ region of the target gene and its upstream nucleotide sequence was amplified using a forward primer containing a 5′ SacI site and a reverse primer containing a 5′ NheI site. For Cj0890c, Cj1024c, and Cj1608, the forward primer contained a BamHI site. For the downstream region of the target gene, an approximately 800-bp fragment that included the 3′ region of the target gene was amplified using a forward primer containing a 5′ NheI site and a reverse primer containing a 5′ SacII site. The 5′ and 3′ fragments of each gene were ligated separately into pCR2.1 using T4 ligase as described by the manufacturer (Invitrogen, Carlsbad, CA). The recombinant plasmids were electroporated into *E. coli* InvαF′, and the recombinant plasmids were purified by cesium chloride centrifugation as outlined elsewhere (33). Following purification, the fragments were ligated into SacI/SacII-digested pBluescript SK(+). In addition, a *C. jejuni* chloramphenicol resistance cassette (800 bp) was cloned into the NheI restriction site. The recombinant vectors were transformed into *E. coli* InvαF′. Constructs from chloramphenicol-resistant colonies were screened to confirm that the NheI chloramphenicol cassette was inserted between the upstream and downstream regions of the target gene. Final mutagenesis vectors were introduced into the *C. jejuni* F38011 wild-type strain by electroporation and selected on MH-blood plates containing chloramphenicol. Putative mutants were confirmed by PCR using flanking upstream and downstream primers, which, due to insertion of the CAT gene, yielded a fragment that was approximately 700 bp larger than the fragment of the *C. jejuni* F38011 wild-type strain.

Complementation of the *C. jejuni cbrR* mutant. The *cbrR* open reading frame with 477 bp of upstream sequence and 60 bp of downstream sequence was amplified from *C. jejuni* strain F38011 genomic DNA using primers CJ0643FH-F (Table 1) and CJ0644FH-R (5′-GCTTCACTGTCTAGATGGCAATG). The PCR product was cloned into the SacI site of pRY107 that harbors a kanamycin resistance cassette (41). The resultant construct harboring the entire *C. jejuni cbrR* gene with its endogenous promoter was designated pBR643. pBR643 was electroporated into the *C. jejuni cbrR* mutant and selected on MH-blood plates containing chloramphenicol and kanamycin. The presence of the shuttle plasmid in the complemented strain, designated the *cbrR*(*cbrR*$^{+}$) mutant, was confirmed

TABLE 1. Oligonucleotide primers used in this study

Primer	DNA sequence (5′ → 3′)[a]
CJ0285FH-F	GAGCTCCATACAAGTAAAGGTAATAAT
CJ0285FH-R	GCTAGCTATAACCACTCCACGAAGATC
CJ0285BH-F	GCTAGCGCTAAAACCTAGAGTTATTAT
CJ0285BH-R	CCGCGGCTCTACTAAATCATGGTCGA
CJ0355FH-F	GAGCTCAATTCATACACATGCACCCG
CJ0355FH-R	GCTAGCTAATGCCGATAAAGTATTCTCC
CJ0355BH-F	GCTAGCCTCCAAGAACTTCAGTTATG
CJ0355BH-R	CCGCGGCTCTATCTCTATGTTTGAGTT
CJ0643FH-F	GAGCTCAAAATTTAAGTTCGGGTGA
CJ0643FH-R	GCTAGCCATTAACTCCTTAGCTTCAGC
CJ0643BH-F	GCTAGCTGGTGAAGTTGTGGATTATG
CJ0643BH-R	CCGCGGGCACTATACCTACCTACAAG
CJ0890FH-F	TTGGATCCTTGCAAAAATGAAAGATGG
CJ0890FH-R	AAAGCTAGCTAAAGATATAACTTCATAACC
CJ0890BH-F	TTGCTAGCGATAAAACAATCAATATAGC
CJ0890BH-R	AACCGCGGGCTGAGTATTTTTTAAAGTG
CJ1024FH-F	AAGGATCCCAAAAAGAGCAGCGATTACAG
CJ1024FH-R	AAAGCTAGCCTTAAAGTAGCATTTCCTGTC
CJ1024BH-F	TTGCTAGCTGGTAATATTAGAGAATTAATATCC
CJ1024BH-R	AACCGCGGAACAGGCACTCTAACACAAGTTGC
CJ1118FH-F	GAGCTCGTCGTTTGGTATATTTTTGC
CJ1118FH-R	GCTAGCCGTGTCCAAGTCTTGTTAGG
CJ1118BH-F	GCTAGCTGAAGCTTGGGATTTATTAAC
CJ1118BH-R	CCGCGGAAAATTCCACTACCACAACC
CJ1223FH-F	GAGCTCAGTATGTGTTCTAAAAGCCA
CJ1223FH-R	GCTAGCCTTCATATCCCTCATCGTTT
CJ1223BH-F	GCTAGCGGATTTTAGATGTAAAAGTTCC
CJ1223BH-R	CCGCGGACTTCTTCATTGCTAGCATGTC
CJ1227FH-F	GAGCTCCCTATTGATCTTAAAAATTATATAG
CJ1227FH-R	GCTAGCCCAAATATTCAGCTGTAATTTCTGC
CJ1227BH-F	GCTAGCGAATATCAGCTTATCATTTTAGACC
CJ1227BH-R	CCGCGGCATTGTTAACCTCAACCCTAGCT
CJ1261FH-F	GAGCTCTACAAGAGCATTTATAAGTAAAGTC
CJ1261FH-R	GCTAGCATATTAAATTGCGCTAAATATTCAG
CJ1261BH-F	GCTAGCATGGTCTTGAAGTTTGTAGAG
CJ1261BH-R	CCGCGGAAAGAAGAAAGAGTACAAAGCTC
CJ1491FH-F	GAGCTCTCATGTAAATATTAATATTGTTTCG
CJ1491FH-R	GCTAGCTCCGATTACTTTACTAAAGCG
CJ1491BH-F	GCTAGCGGCAAGAGAGATTAAAGAAATTTC
CJ1491BH-R	CCGCGGTATAATACCAAGTTGCCCAAATC
CJ1608FH-F	TTGGATCCTGATACCAGAGCAGAATCCTTAAAAA AAGCC
CJ1608FH-R	AAAGCTAGCTCATCAAACGAGTTAATAATTTCAC
CJ1608BH-F	TTGCTAGCAAATATATTTCCTGATACTGATC
CJ1608BH-R	AACCGCGGGCAAATGGTGATGACAACC

[a] The overlined sequences are restriction enzyme recognition sites. Most of the FH-F primers encode a 5′ SacI site; the exceptions are CJ0890FH-F, CJ1024FH-F, and CJ1608FH-F, which encode a BamHI site. The FH-R primers encode a 5′ NheI site, the BH-F primers encode an NheI site, and the BH-R primers encode a SacII site.

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by extraction of the plasmid and subsequent transformation into *E. coli* InvαF′. Kanamycin-resistant *E. coli* InvαF′ transformants were subjected to alkaline lysis, and the extracted plasmid was compared to pBH643 by restriction enzyme analysis.

Bacterial growth assays. In preliminary assays, concentrations of deoxycholate greater than 1% (wt/vol) were found to be inhibitory for the *C. jejuni* F38011 strain. Therefore, suspensions of *C. jejuni* (3 μl of suspensions having an optical density at 540 nm [OD_{540}] of 1.0) were spotted onto MH agar plates supplemented with 1% (wt/vol) sodium deoxycholate to identify the mutants that displayed deoxycholate sensitivity. After 48 h of incubation at 37°C in a microaerobic atmosphere, the presence or absence of *C. jejuni* growth was recorded.

Growth assays were performed using the *C. jejuni* F38011 wild-type strain, the *cbrR* isogenic mutant, and the complemented *cbrR*(*cbrR*$^+$) strain. In these experiments, bacteria were initially inoculated into MH broth to an OD_{540} of 0.02 and incubated at 37°C in a microaerobic atmosphere. Aliquots were removed at intervals to determine the OD_{540}. In some experiments, the number of viable bacteria was determined at various times when organisms were grown in MH broth supplemented with sodium deoxycholate (0.5%, wt/vol). Numbers of viable bacteria were determined by plating serial dilutions of the bacterial suspensions and counting the resultant colonies.

Antimicrobial agent sensitivity assays. The MIC of each antimicrobial agent was determined using a 96-well plate in which the antimicrobial agents were twofold serially diluted. Approximately 10^6 bacteria were inoculated into each well, and the plates were incubated microaerobically for 24 h. Following incubation, the growth of each *C. jejuni* isolate was determined by measuring the OD_{540}. The antimicrobial agents tested included sodium deoxycholate (Fluka, Milwaukee, WI), chenodeoxycholate (Sigma, St. Louis, MO), cholic acid (Sigma), ox bile extract (U.S. Biochemicals, Cleveland, OH), sodium dodecyl sulfate (SDS) (Sigma), Triton X-100 (Calbiochem, La Jolla, CA), Tween 20 (J. T. Baker, Phillipsburg, NJ), nalidixic acid (Sigma), tetracycline (Sigma), gentamicin (Gibco), and sodium chloride (J. T. Baker, Phillipsburg, NJ).

Chicken colonization experiments. One-day-old broiler chickens were obtained from a commercial hatchery. The chickens were negative for *C. jejuni* as determined by culturing cloacal swabs. Nonmedicated feed was given to the chickens ad libitum. In trial one, three groups of chickens (15 animals/group) were inoculated orally when they were 3 days old with approximately 10^5 CFU/bird of either *C. jejuni* F38011, the *cbrR* mutant, or the *cbrR*(*cbrR*$^+$) mutant diluted in MH broth. The second trial was performed as described above, except that 10^6 CFU was used to inoculate each bird. Five chickens were sacrificed on days 3, 6, and 9 postinoculation, and the chicken cecal contents were collected, weighed, serially diluted, and plated on MH agar plates which contained *Campylobacter*-specific growth supplements and selective agents (Oxoid, United Kingdom). The selective plates were pretested for suitability to recover the *C. jejuni* F38011 wild-type strain and the *cbrR* mutant from feces before the chicken experiments were performed. Data are expressed as log CFU/g feces. Representative *Campylobacter* colonies recovered from each group of chickens were selected for confirmation of identity by PCR and growth on appropriate antibiotic-containing culture media.

Motility assays. Each *C. jejuni* RR mutant was tested for motility in semisolid MH medium plates containing 0.3% agar. The plates were incubated microaerobically, and zones of growth were measured after incubation for 48 h.

Statistical analysis. In assays involving survival of the *C. jejuni* F38011, *cbrR*, and *cbrR*(*cbrR*$^+$) strains in the presence of deoxycholate, we utilized the Student's *t* test to determine statistical significance at each time. *P* values are indicated below.

RESULTS

Generation of RR mutants. Studies with several pathogenic bacteria, including *Salmonella* spp., *Shigella* spp., and *Vibrio cholerae*, have shown that bile salts induce an adaptive response to the host environment (reviewed in reference 19). In *Salmonella* spp. and *V. cholerae*, TCR systems that control the expression of virulence genes respond to changes in bile salts, which therefore act as host microenvironment-specific signals (reviewed in reference 19). Therefore, based on such work with other pathogenic bacteria, we hypothesized that a TCR system was involved in the adaptation of *C. jejuni* from an environment without bile salts to an environment with bile salts. Analysis of the *C. jejuni* NCTC 11168 genome revealed the presence of 11 putative RRs (31). Also present in this organism is CheA, which contains both an HK domain and an RR domain. We did not generate a *cheA* mutant as the role of this gene in chemotaxis and motility (11, 18, 21) is well established. We were successful in generating mutants with mutations in 9 of the 11 RRs (Table 2).

TABLE 2. Generation and phenotypic analysis of the *C. jejuni* RR mutants

Gene	Recovery of a null mutant	Motility[a]	Deoxycholate resistance[b]
Cj0285c (*cheV*)	+	−	R
Cj0355c	−	ND[c]	ND
Cj0643 (*cbrR*)	+	+	S
Cj0890c	+	+	R
Cj1024c (*flgR*)	+	−	R
Cj1118c (*cheY*)	+	−	R
Cj1223c (*dccR*)	+	+	R
Cj1227c	−	ND	ND
Cj1261 (*racR*)	+	+	R
Cj1491	+	+	R
Cj1608	+	−	R

[a] −, no migration of the *C. jejuni* strain on semisolid MH agar; +, migration of the *C. jejuni* mutant strain similar to that observed with the *C. jejuni* F38011 wild-type strain.
[b] R, *C. jejuni* mutant exhibits deoxycholate resistance; S, sensitive mutant.
[c] ND, not determined.

Mutations in Cj0355c and Cj1227c could not be generated after repeated attempts. It is noteworthy that the Cj0355c product exhibits 60% amino acid identity with the product of a *Helicobacter pylori* gene, HP1043, which binds promoters that are regulated by growth phase (13). Attempts by other investigators to generate a mutation in HP1043 have not been successful, suggesting that this gene may be required for the organism's viability (7). Based on the homology of Cj0355c with HP1043, we speculate that Cj0355c may play a similar role in *C. jejuni*. Mutation of Cj1227 was attempted in several strains (F38011, NCTC 11168, and 81116) without success. However, construction of a merodiploid containing both a wild-type allele and a mutant allele of Cj1227 was possible in *C. jejuni* strain 480, whereas a recombinant containing two mutated alleles was not recovered (M. Emery and J. Ketley, unpublished data). As this mutant retained a wild-type copy of the gene, we did not use it in the current study. Notably, there is no *H. pylori* homolog for Cj1227c.

In agreement with previous reports, Cj0285c (*cheV*), Cj1118c (*cheY*), and Cj1024 (*flgR*) RR mutants were found to be nonmotile on semisolid MH agar (18, 21, 22, 26, 40, 42). A mutant with a mutation in Cj1608 was also nonmotile. All other mutants displayed motility similar to that of the *C. jejuni* F38011 wild-type strain (Table 2).

Identification of a *C. jejuni* deoxycholate-sensitive mutant. As an initial screen for *C. jejuni* mutants with impaired growth in the presence of sodium deoxycholate, bacterial suspensions were spotted onto MH agar plates supplemented with 1% (wt/vol) sodium deoxycholate (Fig. 1). This concentration of sodium deoxycholate reflects a physiologically relevant range of bile salts present in human bile (25). A single mutant, designated the *cbrR* mutant (for *Camplyobacter b*ile *r*esistance *r*egulator), failed to grow on the deoxycholate-supplemented

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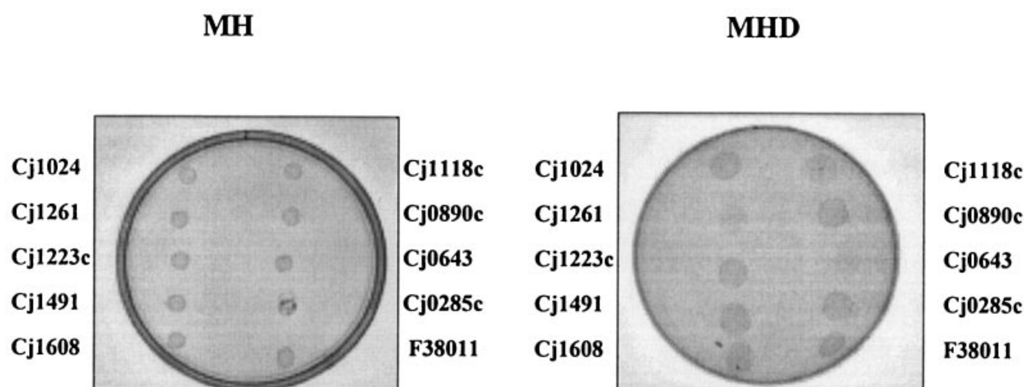

FIG. 1. A *C. jejuni* F38011 *cbrR* (Cj0643) mutant fails to grow on deoxycholate-supplemented MH agar plates. Growth of response regulator mutants on MH agar plates alone or MH agar plates supplemented with 1% sodium deoxycholate (MHD) was assessed by spotting 5 μl of a bacterial suspension which contained approximately 10^6 viable bacteria.

plates and was chosen for further characterization. A complemented *cbrR* strain was constructed by cloning the entire *cbrR* wild-type allele and its native promoter into a shuttle vector (pBR643). Transformation of pBR643 into the *cbrR* mutant resulted in a complemented strain designated the *cbrR*(*cbrR*$^+$) mutant.

To assess whether the *cbrR* mutant had altered growth kinetics when it was grown in sodium deoxycholate, we constructed a growth curve for a 30-h period in the presence or absence of sodium deoxycholate. In preliminary experiments, the addition of various concentrations (0.05 to 1%, wt/vol) of sodium deoxycholate to MH broth was found to decrease the growth rate of the *C. jejuni* F38011 strain compared to the growth rate in MH broth alone. Therefore, we examined the growth kinetics of the *C. jejuni* strains in broth containing a low concentration of deoxycholate (0.05%, wt/vol). Consistent with the results obtained in preliminary experiments, growth of the *C. jejuni* F38011 wild-type strain and the *cbrR* and *cbrR*(*cbrR*$^+$) strains was retarded in MH broth supplemented with 0.05% (wt/vol) sodium deoxycholate (Fig. 2A). However, the growth of the *C. jejuni cbrR* mutant in deoxycholate-supplemented medium was severely impaired throughout the experiment compared to the growth of the *C. jejuni* F38011 wild-type strain. The sensitivity of the *C. jejuni cbrR* mutant to deoxycholate was found to be specific to the *cbrR* gene, as complementation of the mutant with a wild-type copy of the gene in *trans* restored the organism's growth in 0.05% (wt/vol) deoxycholate.

To determine if sodium deoxycholate had a bactericidal or bacteriostatic effect on the *C. jejuni cbrR* mutant, bacteria were cultured in the presence of 0.5% (wt/vol) sodium deoxycholate. This concentration of sodium deoxycholate was chosen to assess the mutant's survival kinetics since the *C. jejuni* F38011 wild-type strain exhibited minimal growth under these conditions. The viability of bacteria at various times was determined by dilution plating. As shown in Fig. 2B, the viability of the *C. jejuni cbrR* mutant steadily decreased over a 20-h experiment. In contrast, the survival of the *C. jejuni* F38011 wild-type strain and the *cbrR*(*cbrR*$^+$) complemented strain after 20 h of incubation was greater than 80% based on the starting inoculum (zero time). A significant difference was noted in the viability of the *C. jejuni cbrR* mutant compared to that of the *C. jejuni* F38011 wild-type strain at 10 and 20 h postinoculation ($P <$ 0.01). These data indicate that the *C. jejuni cbrR* mutant was killed in the presence of deoxycholate in a time-dependent fashion. The rate of killing of the mutant was such that 50% of

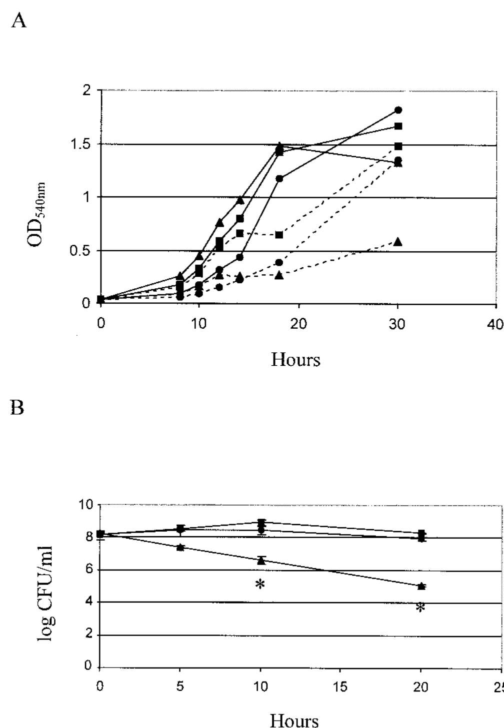

FIG. 2. Effect of sodium deoxycholate on the growth kinetics of *C. jejuni*. (A) Growth (as determined by OD_{540}) of the *C. jejuni* F38011 (■), *cbrR* (▲), and *cbrR*(*cbrR*$^+$) (●) strains in MH broth (solid lines) or MH broth supplemented with 0.05% sodium deoxycholate (dotted lines). The data are from a single experiment that was representative of three independent assays. (B) Survival (expressed as the number of viable bacteria) of *C. jejuni* strains grown in the presence of 0.5% sodium deoxycholate. Statistically significant differences ($P < 0.01$) in the survival of the *C. jejuni* wild-type strain and the *cbrR* mutant are indicated by asterisks. The error bars indicate standard deviations.

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TABLE 3. Sensitivity of *C. jejuni* strains to antimicrobial agents

Antimicrobial agent	MIC for *C. jejuni* F38011 (μg/ml)	*C. jejuni cbrR* mutant		*C. jejuni cbrR*($cbrR^+$) mutant	
		MIC (μg/ml)	Fold difference[a]	MIC (μg/ml)	Fold difference[a]
Sodium deoxycholate	12,500	781	16	12,500	0
Sodium chenodeoxycholate	10,000	1,250	8	10,000	0
Cholic acid	3,125	781	4	3,125	0
Ox bile extract	50,000	6,250	8	25,000	2
Triton X-100	250	62.5	4	125	2
Tween 20	500	62.5	8	250	2
SDS	125	62.5	2	125	0
Nalidixic acid	12.5	12.5	0	12.5	0
Tetracycline	0.04	0.04	0	0.04	0
Gentamicin	0.781	0.781	0	0.781	0
Sodium chloride	12,500	12,500	0	12,500	0

[a] Fold difference in MIC for the *C. jejuni cbrR* or *C. jejuni cbrR*($cbrR^+$) strain compared to the MIC for the *C. jejuni* F38011 wild-type strain.

the initial inoculum survived for 5 h, yet only 0.01% of the inoculum survived for 20 h.

The *C. jejuni cbrR* mutant is sensitive to bile salts. The MICs of various detergents and antibiotics for the *C. jejuni* F38011 wild-type, *cbrR*, and *cbrR*($cbrR^+$) strains were determined (Table 3). Compared to the *C. jejuni* F38011 wild-type strain, the *C. jejuni cbrR* mutant had a lower MIC for all bile salts tested, including sodium deoxycholate, chenodeoxycholate, and cholic acid. More specifically, the MIC of sodium deoxycholate for the *cbrR* mutant was 16-fold lower than that for the wild-type strain. Similarly, the MICs of the nonionic detergents Tween 20 and Triton X-100 were eightfold and fourfold lower for the mutant, respectively. Growth of the *cbrR* mutant was inhibited by 6,250 μg/ml of ox bile extract, while for the wild-type strain the MIC was 50,000 μg/ml. The mutant displayed only a twofold difference in the MIC of SDS, an ionic detergent. In most instances, the MIC for the complemented strain was identical to that for the *C. jejuni* F38011 wild-type strain. For the *C. jejuni cbrR*($cbrR^+$) complemented strain, the MICs of Triton X-100 and Tween 20 were intermediate between the MICs for the *C. jejuni* F38011 wild-type and *cbrR* strains. The MICs of both detergents for the complemented strain were only twofold lower than those for the wild-type strain. No difference was noted for any of the strains for the MICs of the antibiotics tested, including nalidixic acid, tetracycline, and gentamicin. The effect of osmolarity was tested by adding sodium chloride to the medium. The MICs for the strains were identical (12,500 μg/ml), and this concentration of sodium chloride is similar to that reported by other workers as the maximum concentration tolerated for growth of *C. jejuni* (14).

CbrR harbors multiple protein domains. Based on its nucleotide sequence, the *cbrR* gene of *C. jejuni* NCTC 11168 is capable of encoding a 414-amino-acid protein with a calculated molecular mass of 47 kDa. In agreement with the calculated mass, a protein with a similar size was observed (~47 kDa) when the *cbrR* gene from *C. jejuni* F38011 was expressed in *E. coli* BL21 DE3 using the pET expression system (Novagen) (not shown).

As stated above, the *cbrR* gene is not linked with a gene that codes for a cognate HK. In addition, neighboring genes do not provide obvious clues as to its function. For instance, the genes immediately upstream of *cbrR* include Cj0641 (unknown function) and *recN* (encoding a DNA repair protein). Downstream, *cbrR* appears to be translationally linked with Cj0644 (unknown function), Cj0645 (encoding a probable secreted transglycosylase), and Cj0646 (encoding a probable lipoprotein).

Analysis of the CbrR protein demonstrated that it contains two tandem RR receiver domains and a C-terminal GGDEF domain (Fig. 3A). Unlike the archetypical RR, CbrR lacks an obvious DNA-binding motif. As CheY possesses a single well-characterized receiver domain but no DNA-binding domain, we aligned the individual CbrR receiver domains with *E. coli* CheY. As shown in Fig. 3B, receiver domain I shows a high degree of similarity with CheY. Highly conserved residues, such as D12, D13, D57, T87, and K109 (relative to CheY residue numbering), are maintained in domain I (39). Only two residues, D57 and K109, are conserved in receiver domain II (Fig. 3C). Interestingly, both receiver domains contain D57, which is the likely site of phosphorylation (34); however, receiver domain II lacks the D12 and D13 resides that are involved in the active site of CheY. At present, it is not clear whether one or both receiver domains must be phosphorylated for functioning or whether the protein simply phosphorylates another RR.

Analysis of the CbrR protein also revealed a C-terminal GGDEF domain. The GGDEF domain is found in various bacterial signaling proteins and has been proposed to be a diguanylate cyclase (5, 32). For example, two proteins in *Acetobacter xylinum* possess a GGDEF motif, including a diguanylate cyclase and phosphodiesterase A (35). Both proteins are involved in the regulation of cellulose biosynthesis. PleD of *Caulobacter crescentus* exhibits 22% amino acid identity with CbrR and contains two tandem N-terminal RR domains and a C-terminal GGDEF motif. PleD is required for swarmer-to-stalked cell transition (20) and regulates the onset of motility prior to cell division (1). A multiple-sequence alignment of PleD, the GGDEF consensus sequence derived from the Pfam protein domain database (accession number PF00990; http://www.sanger.ac.uk/Software/Pfam/index.shtml), and the GGDEF domain of CbrR is shown in Fig. 3D. While many conserved residues are present in CbrR (including D291, D293, N299, D300, G303, G307, and D308), CbrR aligns poorly with the GGDEF residues at amino acids 332 to 336 (17).

Finally, CbrR lacks an obvious DNA-binding domain and, as

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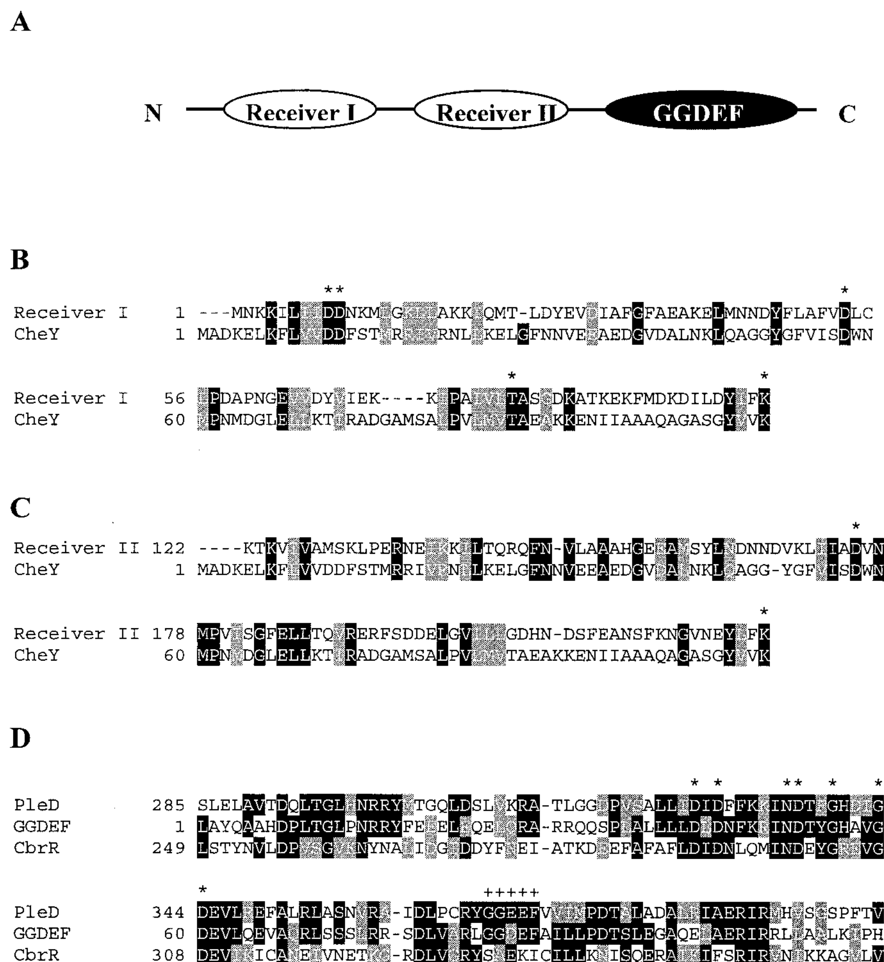

FIG. 3. Analysis of domains present in the CbrR protein. (A) Organization of domains in CbrR. (B) Amino acid sequence alignment of receiver domain I with *E. coli* CheY. (C) Sequence alignment of receiver domain II with *E. coli* CheY. (D) Multiple-sequence alignment of the GGEFD domain of CbrR with PleD (a GGEFD domain-containing protein of *C. crescentus*) and the GGEFD motif consensus sequence (Pfam accession number 00990). The GGDEF residues are indicated by plus signs. The black background indicates identical amino acid residues, and the gray background indicates similar residues. Conserved residues with respect to CheY or the GGDEF consensus sequence are indicated by asterisks.

a result, may interact with another RR as part of a phosphorelay signaling cascade (which ultimately affects the transcription of target genes) or directly with target proteins.

Chicken colonization. Given previous evidence that bile salt resistance contributes to the colonization of chickens (28), we tested the ability of the *C. jejuni cbrR* mutant to colonize chickens. Colonization was compared for the *C. jejuni* wild-type, *cbrR*, and *cbrR*(*cbrR*$^+$) strains. Two chicken colonization experiments were performed. For each experiment, three groups of chickens (15 chickens/group) were inoculated with the *C. jejuni* F38011 wild-type strain, the *cbrR* mutant, or the *cbrR*(*cbrR*$^+$) strain when the birds were 3 days old. At each time, five chickens from each group were sacrificed and tested for *C. jejuni* colonization.

In trial one, chickens were inoculated with 1.6×10^5 CFU of *C. jejuni* F38011, 2.3×10^5 CFU of the *cbrR* strain, and 5.6×10^5 CFU of the *cbrR*(*cbrR*$^+$) strain. On day 3 postinoculation, four of five of the *C. jejuni* F38011-inoculated chickens were colonized (Fig. 4A). The extent of colonization ranged from 6×10^2 CFU/g feces to 6.5×10^4 CFU/g feces. Only one of the chickens inoculated with the *cbrR* mutant was colonized (6.5×10^4 CFU/g feces). On day 6, all of the *C. jejuni* F38011-inoculated chickens were colonized (range, 5×10^3 to 1.0×10^7 CFU/g feces), but none of the chickens inoculated with the *C. jejuni cbrR* mutant were colonized. On day 9, four of five chickens inoculated with the *C. jejuni* F38011 wild-type strain were colonized, while only a single chicken was colonized in the group inoculated with the *cbrR* mutant. One of the birds inoculated with the *C. jejuni cbrR* mutant died prior to necropsy on day 9. In this experiment, the *C. jejuni cbrR*(*cbrR*$^+$) complemented strain failed to colonize chickens over the course of the experiment.

The same experiment was repeated in trial two (Fig. 4B). Three groups of birds were inoculated with 4×10^6 CFU of the *C. jejuni* F38011 wild-type strain, 4×10^6 CFU of the *cbrR* strain, and 2×10^6 CFU of the *cbrR*(*cbrR*$^+$) strain. The *C. jejuni* F38011 wild-type strain-inoculated birds were colonized throughout the experiment at levels comparable to those in trial one (range on day 3, 7×10^2 to 1×10^5 CFU/g feces), while only two birds in the group inoculated with the *cbrR*

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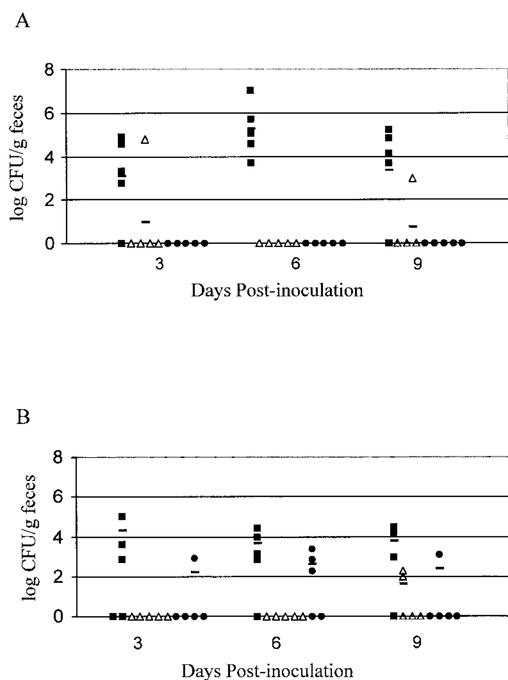

FIG. 4. Colonization of chickens by various *Campylobacter* strains. Cecal contents were collected at necropsy on day 3, 6, or 9 postinoculation. The data in panels A and B are the data from two independent experiments. (A) Trial one. Chickens were inoculated with approximately 10^5 CFU of the *C. jejuni* F38011 (■), *cbrR* (△), or *cbrR*(*cbrR*$^+$) (●) strain. (B) Trial two. Chickens were inoculated with approximately 10^6 CFU of each *C. jejuni* strain. Each symbol indicates the log CFU/g feces isolated from an inoculated chicken at a time postinoculation. The mean log CFU/g feces for each group is indicated by a horizontal bar.

mutant showed a low level of colonization on day 9 (range, 1×10^2 to 2×10^2 CFU/g feces). A single chicken inoculated with the *C. jejuni* wild-type strain died prior to necropsy on day 9. In contrast to trial one, the *C. jejuni cbrR*(*cbrR*$^+$) strain did colonize some of the birds at levels similar to those observed with the *C. jejuni* F38011 wild-type strain.

DISCUSSION

Intestinal pathogens must resist the antimicrobial effects of bile as they pass through the upper small intestine, as well as decreased levels in the lower intestine. The importance of resistance to bile is illustrated by experiments with various enteric bacteria. Bile resistance is required for intestinal colonization in animal models by *Listeria monocytogenes* and *V. cholerae* (8, 15). *Salmonella enterica* serovar Typhi can establish persistent colonization of the gall bladder among carriers, where it must be able to survive high concentrations of bile. *C. jejuni* must be resistant to bile to colonize chickens via the multidrug efflux pump CmeABC (28). Hence, an adaptive response to bile has been shown to be important in virulence, and there is evidence that two-component regulation is involved in regulating this response.

In this study, we set out to generate knockout mutants with mutations in RRs encoded by *C. jejuni* that may affect bile resistance. We confirmed mutations in 9 of 11 RRs targeted. We propose that one of the remaining genes, Cj0355c, may be required for viability based on studies with HP1043 of *H. pylori* (7). The failure to obtain a mutant with a mutation in Cj1227c remains to be explained, but we were able to obtain a Cj1227 merodiploid that retained a copy of the wild-type allele. Consistent with previous reports, we found that *cheV*, *cheY*, and *flgR* mutants were nonmotile on semisolid agar (18, 26, 21, 22, 40, 42). The RR mutants were initially screened for growth on MH agar plates supplemented with 1% sodium deoxycholate, which is a high but subinhibitory concentration of this bile salt. For *C. jejuni*, the MIC of sodium deoxycholate is approximately 1.25%. Only one *C. jejuni* mutant screened in this study, the *cbrR* mutant, failed to grow on this medium. While it is possible that other RR mutants display altered growth kinetics in the presence of high concentrations of sodium deoxycholate, we further examined the *cbrR* mutant as the mutation in the *cbrR* locus had the most pronounced effect.

The *C. jejuni cbrR* mutant was unable to grow in the presence of a high concentration (1%, wt/vol) of sodium deoxycholate, and growth was significantly inhibited at a low concentration (0.05%, wt/vol) of sodium deoxycholate. In contrast to the *C. jejuni* F38011 wild-type strain, the *C. jejuni cbrR* mutant was killed in the presence of sodium deoxycholate over a 20-h experiment. In addition, ox bile extract, which contains a mixture of bile salts, had an inhibitory effect on the growth of the *C. jejuni cbrR* mutant compared to the growth of the wild-type strain. The sensitivity of the *C. jejuni cbrR* mutant appeared to be specific for detergents, and the greatest effect was the effect of bile salts. Several mutations have been reported to affect bile resistance in bacteria, including mutations in genes that encode lipopolysaccharide, porins, efflux pumps, or regulatory genes (19). Such mutations may affect membrane access (lipopolysaccharide) or transport of bile salts (porins and efflux pumps). Regulatory genes, such as the genes encoding *E. coli* RRs EvgA and BaeR, control expression of the multidrug efflux pumps EmrKY and MdtABCD, respectively (6, 30). The PhoPQ system is required for resistance to bile but not for resistance to SDS or Triton X-100 in *Salmonella* spp. (38). While bile affects the synthesis of many *Salmonella* proteins, including those regulated by PhoPQ, no proteins were found to mediate bile resistance directly.

In *C. jejuni*, the multidrug efflux pump, CmeABC, confers resistance to a variety of antimicrobial agents, including bile salts, antibiotics, and toxic compounds such as ethidium bromide (27). CmeABC is subject to regulation by a local transcriptional repressor, CmeR, which is encoded by a gene immediately upstream of *cmeABC* (28a). CmeR binds specifically to the inverted repeat sequences in the promoter of *cmeABC* and modulates the expression of this efflux pump. It is noteworthy that the promoter activity of *cmeABC* is bile salt inducible and that induction is dependent on CmeR (Q. Zhang, unpublished data). While there is no evidence that bile salts affect CmeR binding, additional work is required to determine if CbrR interacts with CmeR to modulate expression of CmeABC. The mechanism of *C. jejuni* CbrR in the control of bile resistance remains to be determined.

CbrR does not have an adjacent partner HK but may become phosphorylated by one or more HKs encoded by *C. jejuni.* Therefore, it is possible that at least one HK mutant could display a deoxycholate-related phenotype. In a phos-

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phorelay system, it is also likely that one or both of the receiver domains of CbrR can transfer a phosphate to an alternate RR or HK. However, it is not known whether CbrR transfers a phosphate to a partner HK or RR. It is also possible that one or both RR domains control the activity of the GGDEF domain in a manner similar to the phosphorylation of the RR domain of CheB, which, in turn, activates a C-terminal methylesterase domain (3). Alternatively, CbrR may interact directly with another protein. Using a yeast two-hybrid approach, at least 19 proteins that interact with CbrR have been identified (E. Dave and J. Ketley, Abstr. 104th Gen. Meet. Am. Soc. Microbiol., abstr. H-059, 2004). It is not known whether mutations in the target genes encoding these proteins result in phenotypes similar to that of the *C. jejuni cbrR* mutant. Identification of other members of the CbrR regulon will likely aid in characterization of the physiology of bile resistance in *C. jejuni*. Target proteins with which CbrR interacts or proteins whose expression is regulated by an RR that functions downstream of CbrR would likely play a more direct role in bile resistance.

The GGDEF domain has been identified in a number of multidomain regulatory proteins (17). The function of this domain has been reported to be a diguanylate cyclase containing a putative nucleotide-binding loop (32, 35). Reports of other proteins with a domain architecture similar to that of CbrR (RR-RR-GGDEF) indicate that such proteins do not have similar functions. One RR, PsfR of the cyanobacterium *Synechococcus elongatus*, is involved in the regulation of circadian expressed genes (37). The N-terminal RR domain of this protein lacks the conserved aspartyl residue required for phosphorylation. Conversely, *C. crescentus* PleD lacks the aspartyl residue in the C-terminal RR domain (20). Unlike either of these proteins, CbrR contains an aspartyl residue in both RR domains, suggesting that one or both domains may be phosphorylated. Another RR, RrpX of *Aeromonas jandaei*, allows overexpression of the *A. jandaei* β-lactamase, AsbB1, in *E. coli* but not in *A. jandaei* (2). Using a conserved domain database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd), we found that RrpX also contains the RR-RR-GGDEF architecture. Both RR domains appear to have the aspartyl residue required for phosphorylation; however, the N-terminal RR domain lacks a D12 residue, and the C-terminal RR domain lacks a critical lysine residue (K109).

The *C. jejuni cbrR* mutant has a reduced ability to colonize chickens, which may be due to its sensitivity to bile salts. Lin et al. (28) reported that the concentration of bile salts in the chicken intestine ranges from 0.085 mg/ml in the cecum to 7 mg/ml in the jejunum. In the present study, chicken colonization was assessed by sampling cecal contents, where the concentration of bile is reportedly low; however, *C. jejuni* must pass through the upper intestine, where the bile salt concentration is higher than the MIC observed for the *cbrR* mutant (~1 mg/ml). We speculate that the high concentration of bile in certain regions of the chicken digestive tract is sufficient to kill the mutant before it colonizes the cecum. While not all birds were colonized by the *cbrR*(*cbrR*$^+$) complemented strain at a given time, those that were colonized had colonization levels similar to that of birds inoculated with the *C. jejuni* F38011 wild-type strain. The reason for the decreased number of birds colonized by the complemented strain is unclear, as its resistance to bile salts is similar to that of the *C. jejuni* F38011 wild-type strain. It is noteworthy that for the complemented strain the MICs of nonionic detergents were intermediate between those for the *C. jejuni* F38011 wild-type strain and the *cbrR* mutant. Hence, the complemented strain may retain sensitivity to in vivo factors that were not tested in our in vitro assays.

The identification of CbrR as a regulator of bile resistance in *C. jejuni* is consistent with previous reports which showed that bile resistance is required for chicken colonization (28). Future studies will require identification and functional characterization of a CbrR-protein partner(s) that affects bile resistance in *C. jejuni*.

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