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A Genetic Locus Involved in Iron Utilization Unique to Some *Campylobacter* Strains

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Two genes involved in iron utilization in *Campylobacter coli* VC167 T1 have been characterized. The *cfrA* gene encodes a protein with a predicted M_r of 77,653 which, after processing of the leader sequence, has a predicted M_r of 75,635. This protein has significant sequence identity to siderophore receptors of several bacteria, and site-specific mutants defective in *cfrA* do not synthesize one of two major iron-repressible outer membrane proteins. An adjacent gene encodes a TonB-like protein; a mutant in this gene lost the ability to utilize hemin, ferrichrome, and enterochelin as iron sources. The *cfrA* and *tonB* genes of VC167 T1 hybridized to all strains of *C. coli* and most strains of *C. jejuni* examined but did not hybridize to several other strains of *C. jejuni*, suggesting that the thermophilic campylobacters can be separated into two categories based on the presence of these two iron utilization genes.

The thermophilic *Campylobacter* species, *C. jejuni* and *C. coli*, are the most frequently isolated bacterial causes of diarrheal disease in the world (54, 57). However, the virulence mechanisms in this genus are less well understood than those of some of the less frequently isolated causes of bacterial diarrhea, and only a cursory outline of the disease process has evolved. The organisms are characterized by a rapid darting motility, and this motility is necessary for colonization of the gastrointestinal tracts of various animals and humans (8, 36, 42). A bile acid-inducible pilus has recently been identified in *Campylobacter* spp. (12). Nonpiliated mutants of *C. jejuni* are able to colonize animals but unable to cause disease in a ferret diarrheal disease model (12). This finding suggests that pili may be induced in the bile-rich environment of the small intestine and mediate tropism of the bacteria to target cells in the colon (8, 12), where disease ensues. The mechanism by which diarrhea develops is unknown, but cytotoxins (29, 44) and invasive mechanisms may be involved (20, 31, 40, 57).

Pathogenic bacteria are equipped with two general iron uptake mechanisms (11, 21): the production of siderophores, small iron scavenger molecules with high affinity for iron bound to transferrin or lactoferrin, and the direct utilization of host iron compounds, such as transferrin or hemin. Among the bacterial species that produce siderophores are *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Vibrio cholerae*, *Yersinia enterocolitica*, and *Vibrio anguillarum* (11, 21). In all of these bacteria, free-iron-restricted growth conditions induce expression of genes needed for the synthesis of both the siderophore and outer membrane proteins that act as receptors for the iron-siderophore complex. The bound iron-siderophore complex is transported across the outer membrane by a TonB-dependent mechanism (21). TonB proteins, which are thought to be anchored in the inner membrane and span the periplasmic space, function to provide energy to the outer membrane for the transport of numerous substrates into the periplasmic space, including vitamin B_{12}, bacteriocins, and iron compounds (13).

Among the pathogens that are able to utilize hemin as a source of iron are *Neisseria gonorrhoeae* (38), *Haemophilus influenzae* (10, 24), *Y. enterocolitica* (51, 52), *V. cholerae* (50), *Legionella pneumophila* (39), and *C. jejuni* (43). Utilization of hemin is best understood in *Y. enterocolitica*. The hemin receptor of *Y. enterocolitica* has been identified as HemR, an iron-regulated outer membrane protein with an M_r of 78,000 that has homology with the vitamin B_{12} receptor of *E. coli* (51, 52). In *V. cholerae*, *hutA*, a gene encoding an iron-regulated outer membrane protein, has been shown to be needed for uptake of hemin (50), and hemin uptake has also been shown to be TonB dependent (52).

Iron availability for bacterial assimilation within the human host is limited since most iron is sequestered by high-affinity binding proteins such as transferrin and lactoferrin. Although the ability to compete successfully for iron in vivo is generally considered to be a virulence determinant of bacterial pathogens, the value of iron uptake systems for pathogens which grow in the environment of the intestinal tract is less clear. Mutants defective in iron uptake systems in *Salmonella typhimurium* (6) or *V. cholerae* (49) are not reduced in virulence. However, iron uptake mutants of *S. flexneri* are attenuated (35), and such mutations have been included in vaccine strains currently in human testing (3, 48).

Comparatively little is understood about iron uptake in *Campylobacter* spp. Only a few strains of *Campylobacter* have been shown to synthesize siderophores (14), but they are able to utilize enterochelin, a siderophore produced by *E. coli* and other enteric bacteria, and ferrichrome, made by *Ustilago sphaerogena* (2, 14). The periplasmic components of the enterochelin uptake system have been cloned from strains of both *C. jejuni* (41) and *C. coli* (46). Pickett et al. (43) have reported that *C. jejuni* 81-176 is capable of hemin and hemoglobin uptake and have shown that an iron-repressible outer membrane protein with an M_r of approximately 71,000 is required, but this putative receptor has not been characterized further.

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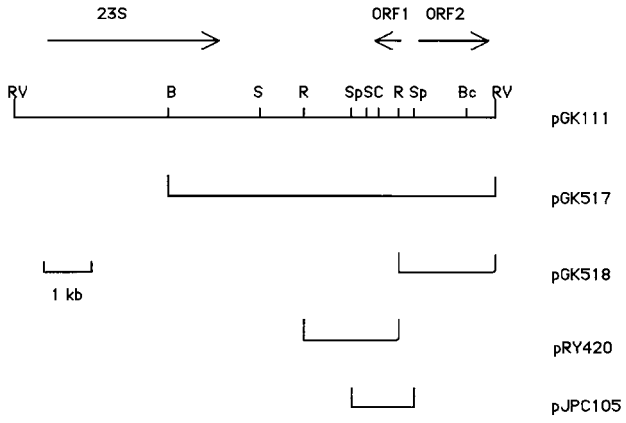

FIG. 1. Schematic representation of the DNA cloned in plasmid pGK111, which contains *C. coli* VC167 T1 DNA encoding a 23S rRNA gene cloned into the *Eco*RV site of pBR322 (50), and relevant subclones or deletion derivatives. Plasmid pGK517 is a deletion from the *Bam*HI site within the 23S gene to the *Bam*HI site within pBR322. Plasmids pGK518 and pRY420 are subclones of pGK517 in pUC18, and pJPC105 is a subclone of pGK517 in pUC19. The arrows indicate the direction of transcription of the 23S gene and the two ORFs described in this report. RV, *Eco*RV; B, *Bam*HI; S, *Spe*I; C, *Cla*I; Bc, *Bcl*I; R, *Eco*RI; Sp, *Ssp*I.

In this report we describe two genes, isolated from *C. coli* VC167 T1, which are apparently involved in utilization of iron. One of these genes encodes an iron-repressible outer membrane protein with significant sequence similarity to siderophore receptors of *Bordetella bronchiseptica* and *V. cholerae*. The other gene encodes a TonB-like protein which is required for utilization of hemin, ferrichrome, and enterochelin. In addition, we show that these genes are present in all strains of *C. coli* and most strains of *C. jejuni* examined but are not present in several of the better-characterized *C. jejuni* strains, including 81-176 (8, 32, 40, 43, 44) and 81-116 (20, 41, 57).

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. coli* VC167 T1 has been described previously (26). *E. coli* DH5α was used as the host for cloning experiments; pBR322, pUC18, pUC19, and pBluescript were used as cloning vectors, and pILL600 (33) was the source of the kanamycin resistance (Km^r) cassette.

Media and growth conditions. *Campylobacter* spp. were routinely grown on Mueller-Hinton (MH) agar (Difco, Detroit, Mich.) under microaerobic conditions. *E. coli* was grown on LB (Gibco, Gaithersburg, Md.). When appropriate, kanamycin was added to a final concentration of 50 μg/ml and ampicillin was added to a final concentration of 100 μg/ml.

DNA methods. Restriction enzymes, T4 DNA ligase, and DNA-modifying enzymes were purchased from Boehringer Mannheim Biochemicals and used as recommended by the supplier. Plasmid DNAs were routinely isolated by using Qiagen columns (Qiagen, Chatsworth, Calif.) or by an alkaline lysis procedure (7) followed by cesium chloride-ethidium bromide gradient centrifugation. Total campylobacter DNAs were isolated by the method of Alm et al. (1). Probes for hybridization were labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.), using commercial kits, by either nick translation (New England Nuclear) or random priming (Boehringer Mannheim). DNA hybridizations were done in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C and washed in 0.5× SSC at 60°C (23). These wash conditions correspond to a temperature 16°C below T_m.

Inverse PCR was used to amplify the DNA downstream of open reading frame 2 (ORF2) (Fig. 1). Total VC167 T1 DNA was digested with *Bcl*I and treated with Strataclean resin (Stratagene, La Jolla, Calif.). The DNA was self-ligated at a final DNA concentration of 10 μg/ml, extracted with phenol-chloroform, and ethanol precipitated. After resuspension in TE (0.01 M Tris, 0.001 M EDTA [pH 8]), an aliquot was used as the template in a PCR using reagents from Perkin-Elmer/Applied Biosystems (Foster City, Calif.). The primers were cp1 (5′-CCT TGGATAAAAGGCGAG-3′) and cp2 (5′-ATCAGCATCACAAGTGCC-3′). Both primers bind with ORF2 between the *Bcl*I and *Eco*RV sites in Fig. 1; cp1 binds between bp 2887 and 2904, and cp2 binds on the other strand between bp 2661 and 2644 on the sequenced DNA as deposited in GenBank (see below). The PCR conditions were 94°C for 30 s, 50°C for 30 s, and 70°C for 2 min for 30 cycles. The 1.3-kb product was sequenced directly by using cp1, cp2, and custom primers.

DNA sequencing. DNAs were sequenced by using dideoxy terminator *Taq* cycle sequencing kits from Perkin-Elmer/Applied Biosystems and analyzed on an Applied Biosystems model 373 automated DNA sequencer.

In vitro transcription-translation. Purified plasmid DNAs were used as templates in in vitro transcription-translation assays using a commercial kit (Promega, Madison, Wis.) and [^{35}S]methionine (Amersham, Oakville, Ontario, Canada). Aliquots of the reactions were precipitated with acetone and dissolved in protein loading buffer (30), and the proteins were separated on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels. The gels were dried and exposed to film for 24 h at −70°C.

Protein electrophoresis and N-terminal amino acid sequencing. Protein outer membrane fractions of *C. coli* strains were prepared as described previously (16) from cells grown in the presence or absence of the iron chelator ethylenediamine di(*O*-hydroxyphenyldiacetic acid) (EDDHA). Proteins were separated on SDS–10% polyacrylamide gels and stained with Coomassie blue R250.

For N-terminal amino acid sequence analysis, proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to a ProBlott membrane (Applied Biosystems, Foster City, Calif.) as specified by the manufacturer. Amino acid sequence analysis was performed on an Applied Biosystem model 470A gas-phase sequenator using a standard operating program.

Bioassays. The ability of *C. coli* strains to utilize a 1-mg/ml hemin solution, enterochelin (a generous gift of Luis Actis and Victor de Lorenzo), ferrichrome (a generous gift of Victor de Lorenzo and Jorge Crosa), 100 mM $FeSO_4$, and 100 mM ferric citrate as sources of iron was tested as follows. Overnight plate cultures of the strains were resuspended in MH broth with sterile cotton swabs. The optical density of the cell suspensions was determined, and then the suspensions diluted to an optical density at 600 nm of 1.0. One milliliter of each diluted cell suspension was mixed with 20 ml of MH agar containing 200 μM the iron chelator EDDHA and poured into petri dishes. A 5-μl aliquot of the different iron sources was spotted on top of the solidified agar surface, and the plates were incubated overnight at 37°C under microaerobic conditions. The ability of the strains to utilize the different iron sources was determined by the presence of a halo of growth around the spots.

Nucleotide sequence accession number. The DNA sequence of ORF1, ORF2, and the intergenic region shown in Fig. 1 has been deposited in GenBank under accession no. U80812.

RESULTS

Molecular analyses. Plasmid pGK111 has previously been reported to contain a copy of a 23S ribosomal gene from VC167 T1 on a 9-kb *Eco*RV fragment in pBR322 (55). Additional sequence analysis at the opposite end of this insert revealed two adjacent ORFs on opposite strands, as shown in Fig. 1.

ORF1 encoded a predicted protein which was 232 amino acids in length, with a predicted molecular weight of 26,989 and a pI of 9.76. The predicted protein, which contained 42.3% hydrophobic residues, 18.6% basic residues, and 8.7% acidic residues, showed no significant matches to any known proteins by BLASTP or BLITZ analyses. However, among the proteins which showed the highest homology scores were TonB proteins from several bacteria. Examination of the ORF1 gene product revealed several features typical to TonB proteins, including a high proline content. While TonB of *E. coli* (M_r, 26,656; pI = 9.5) has one of the higher proline contents of TonB proteins (17% [45]), TonB of *H. influenzae* (M_r, 28,394; pI = 9.1) has a proline content of 8% (28), as does the product of ORF1. TonB proteins are also characterized by numerous glutamate-proline and lysine-proline repeats. The ORF1 product contains fewer such repeats than known TonB proteins, but some are present in similar positions of the molecule (Fig. 2). The amino-terminal region of the predicted protein has properties similar to those of a signal sequence but lacks a signal peptidase cleavage site, similar to known TonB proteins (13). The algorithm of Klein et al. (30) predicts this region of the protein to be a membrane-spanning region, which is also characteristic of TonB proteins (13). Figure 2 shows an alignment of the carboxy-terminal region of *H. influenzae* TonB (28) with the gene product of ORF1; the two proteins show 21% identity and 41% similarity over a region of 174 amino acids. For

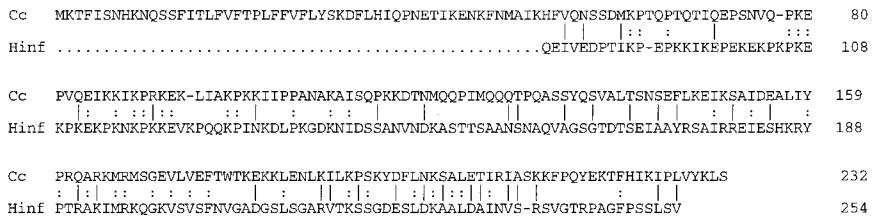

FIG. 2. Comparison of the protein encoded by ORF1 (Cc) with the carboxy-terminal portion of the TonB protein of *H. influenzae* (Hinf). The colons indicate identical residues, and the dashes indicate conserved residues. The amino acid number of each protein is shown on the right.

comparison, the TonB proteins of *H. influenzae* and *E. coli* show 22% identity and 49% similarity over a stretch of 213 amino acids (not shown). Clustal phylogenetic tree analysis (27) (data not shown) indicates that the campylobacter protein diverged earlier than the TonB proteins from members of the family *Enterobacteriaceae.*

ORF2 encoded a protein of >556 amino acids, but the ORF extended beyond the *Eco*RV site marking the end of the cloned DNA (Fig. 1). Attempts to clone overlapping, downstream pieces of DNA repeatedly resulted in deletions and/or rearrangements. The remaining sequence of the ORF was determined by direct sequencing of an inverse PCR product which overlapped pGK517 by 732 bp and which was generated as described in Materials and Methods. The full-length ORF2 encoded a protein with a predicted M_r of 77,653 and a pI of 5.07. This protein has 35.2% hydrophobic residues, 10.6% basic residues, and 11.6% acidic residues. The protein is predicted to have 8.8% helical and 28.7% extended structure (47), and the algorithm of Klein et al. (30) indicates that it is a membrane protein. There is a putative leader sequence and signal peptidase cleavage site (see below), and the carboxy-terminal 10 amino acids are consistent with those of outer membrane proteins (53). BLASTP analysis of this protein showed significant sequence similarity to BfrA, a putative exogenous ferric siderophore receptor of *Bordetella bronchiseptica* (4), and a lesser (though still significant) homology to IrgA of *V. cholerae*, a protein which binds ferric vibriobactin (17–19). Comparison of the ORF2 gene product with BfrA is shown in Fig. 3. The campylobacter protein shows 35% identity and 56% similarity to BfrA over a region of 698 amino acids. Although there is homology throughout the proteins, the highest similarity is at the N-terminal region. Over a region of 188 amino acids at the N terminus, the ORF2 gene product shows 50% identity and 74% similarity with BfrA. The similarity to IrgA is also stronger at the amino-terminal ends of the two proteins. Over a region of 221 amino acids at the N terminus, CfrA shows 36% identity and 71% similarity to IrgA (data not shown). Based on this sequence similarity and data indicating that this protein is iron regulated as shown below, we have tentatively named this gene *cfrA* (campylobacter ferric receptor). Both BfrA and IrgA contain sequences resembling the consensus TonB pentapeptide box (ETVIV) at their N termini, as indicated for BfrA by the overlining in Fig. 3. The same region of CfrA contains a related but variant pentapeptide (DSSII). However, genetic data for *E. coli* indicate that TonB interacts with more of TonB-dependent proteins than just the TonB box (5).

The two ORFs are separated by 321 bp; this 321-bp region presumably contains the promoters of each gene in opposite orientations. The overall G+C content of the intergenic region is 17%, while the total G+C content of the DNA sequenced is 31%. There are two potential Fur boxes (5′-TTAATTAATA CTAATTATC-3′ and 5′-TTAAATTAATAATTATTATC-3′) starting 86 and 45 bp, respectively, 5′ to the start of the *cfrA*

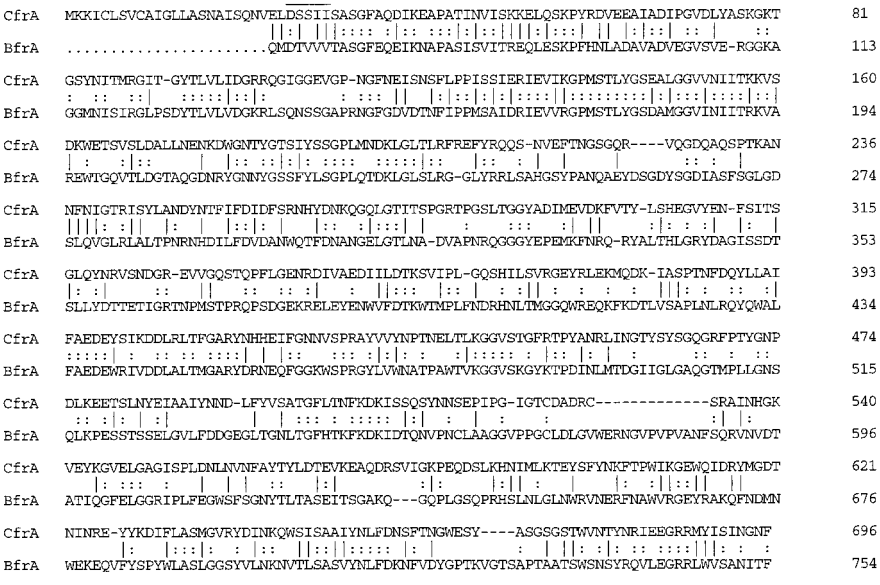

FIG. 3. Comparison of the first 527 amino acids of the protein encoded by ORF2 with residues 56 to 754 of the BfrA protein of *B. bronchiseptica* (3). The colons indicate identical residues, and the dashes indicate conserved residues. The amino acid number of each protein is shown on the right.

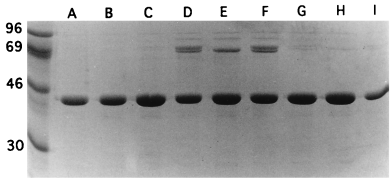

FIG. 4. Outer membrane protein profiles of *C. coli* strains grown under different conditions and separated on SDS–10% polyacrylamide gels. Lane A, VC167 T1 grown in MH; lane B, PG1024 grown in MH; lane C, PG1093 grown in MH; lane D, VC167 T1 grown in MH–50 μM EDDHA; lane E, PG1024, grown in MH–50 μM EDDHA; lane F, PG1093 grown in MH–50 μM EDDHA; lane G, VC167 T1 grown in MH–50 μM EDDHA–50 μM ferric citrate; lane H, PG1024 T1 grown in MH–50 μM EDDHA–50 μM ferric citrate; lane I, PG1093 T1 grown in MH–50 μM EDDHA–50 μM ferric citrate. The first lane contains size markers, positions of which are indicated on the left in kilodaltons.

gene (data not shown) (9, 13, 58), but there are no obvious Fur boxes in the region closer to the start of the *tonB*-like gene.

Analyses of site-specific mutants. To explore the potential role of the proteins encoded by *cfrA* and the *tonB*-like gene on iron uptake, site-specific mutants were isolated by standard methodology. A *Bam*HI-ended Km^r cassette was inserted into a unique *Bcl*I site within *cfrA* on pGK518 to generate pGK518::Km, and this DNA was used to transform VC167 T1 to Km^r. To mutate the *tonB*-like gene, an *Acc*I-ended Km^r cassette was inserted into a unique *Cla*I site in pRY420 (Fig. 1) to generate a plasmid called pRY420::Km. Attempts to introduce this plasmid into VC167 T1 by natural transformation were unsuccessful; therefore, the insert was transferred into the *Sma*I site on the suicide vector pGK2003 (22) as a *Pvu*II fragment and conjugally mobilized into VC167 T1 by using RK212.1 as the donor (15), with selection for Km^r. All potential mutants were screened by hybridization with the cloning vector to ensure that no vector DNA had integrated via a single crossover, and putative mutants were analyzed by hybridization to confirm that predicted double crossover of the mutated allele had occurred (data not shown). The VC167 T1 mutant defective in the *cfrA* gene was called PG1024, and the mutant defective in the *tonB*-like gene was called PG1093.

VC167 T1, PG1024, and PG1093 were compared for the ability to utilize different iron compounds by bioassays. The results indicated that VC167 T1 and the *cfrA* mutant, PG1024, were able to utilize hemin, ferrichrome, and enterochelin as sources of iron. The halos of growth around each of these compounds were the same size in the wild type and the *cfrA* mutant. However, the *tonB* mutant, PG1093, had lost the ability to use hemin, ferrichrome, and enterochelin. All three strains showed halos of the same size with $FeSO_4$ and ferric citrate.

Protein analyses. When VC167 T1 was grown in medium containing EDDHA, an increase in the expression of two major outer membrane proteins with apparent M_rs of 71,000 and 75,000 were seen on SDS-polyacrylamide gels (Fig. 4, lanes A and D). Growth of the *cfrA* mutant strain PG1024 in the same conditions resulted in expression of only the protein with an apparent M_r of 71,000 (Fig. 4, lanes B and E). This finding suggests that the outer membrane iron-repressible protein with an apparent M_r of 75,000 corresponds to CfrA. N-terminal amino acid sequencing of the protein with an apparent M_r of 75,000 indicated that the N terminus corresponded to amino acids 21 to 30 (QNVELDSSII) of the predicted CfrA protein. This result confirms that the observed protein with an apparent M_r of 75,000 is CfrA and indicates that the predicted M_r of the mature protein after cleavage of the leader sequence should be 75,648, which is consistent with that observed experimentally. Addition of iron in the form of ferric citrate represses expression of the two proteins in VC167 T1 (Fig. 4, lane G) and the remaining protein in the *cfrA* mutant (Fig. 4, lane H).

The outer membrane profile of the *tonB* mutant, PG1093 (Fig. 4, lanes C and F), grown in MH did not differ from that of VC167 T1 (Fig. 4, lanes A and D), indicating that under these growth conditions, the *tonB* mutant is not iron starved. This would suggest that TonB-independent uptake systems similar to those seen in anaerobically grown *E. coli* (25) are operable in this strictly microaerophilic organism. Chelation of the iron results in derepression of the two proteins in the *tonB* mutant (Fig. 4, lane F), and when ferric citrate was added to the medium, the expression of the iron-inducible outer membrane proteins was also reduced (Fig. 4, lane I).

To determine if the *cfrA* and *tonB* genes were expressed in *E. coli*, in vitro transcription-translation experiments were carried out with plasmids pGK518, pGK518::Km, pJPC105, and pRY420 as templates (Fig. 1). The results are shown in Fig. 5. Among the bands observed by in vitro translation of pGK518 (lane A) are a protein with an apparent M_r of 71,000 which presumably corresponds to the truncated form of CfrA (predicted M_r of 71,320) encoded on pGK518 (indicated by the closed circle). The M_r-71,000 protein is missing in pGK518::Km in which the *cfrA* gene has been insertionally inactivated (lane B). Instead, pGK518::Km encodes a protein with an approximate M_r of 44,000 (indicated by the open circle). Insertion of the Km^r cassette into the *Bcl*I site (located 1,158 bp into the coding region of the gene) would be expected to result in a truncated CfrA protein with an M_r of 42,219. The protein with an apparent M_r of 31,000 (indicated by the open arrowhead) in lane B is likely the Km^r protein, which has a predicted M_r of 30,971 (33). The protein with an apparent M_r of 27,000, designated by the asterisk in lane C (pJPC101), most likely represents the product of the *tonB* gene, based on its size and its absence in the in vitro translation products obtained by using as the template plasmid pGK518 (lane A) or pRY420 (lane D), in which all or part of *tonB* has been deleted. The major band at an M_r of 31,500 in all four lanes (indicated by the closed arrowhead) presumably is the β-lactamase of the vector.

DNA hybridization studies. Two probes were used to determine the presence of *tonB* and *cfrA* in other strains of *Campylobacter* by either Southern or colony blot analyses. The probes were pGK518, containing the *cfrA* gene, and a 291-bp *Cla*I-*Eco*RI fragment internal to the *tonB* gene from plasmid pRY420 (Fig. 1). Representative results are shown in Fig. 6. Both probes hybridized to six of six strains of *C. coli* other than VC167, and all were clinical isolates from Canada. Both probes

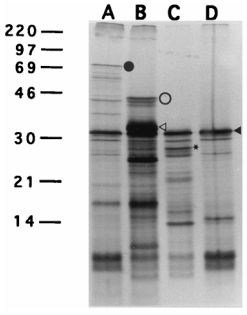

FIG. 5. In vitro transcription and translation of plasmid DNAs. Plasmid-encoded proteins were labeled with [^{35}S]methionine using a Promega in vitro transcription-translation kit and separated on an SDS–12.5% polyacrylamide gel. Lane A, pGK518; lane B, pGK518::Km; lane C, pJPC105; lane D, pRY420. Positions of size standards are indicated on the left in kilodaltons. For explanation of symbols, see Results.

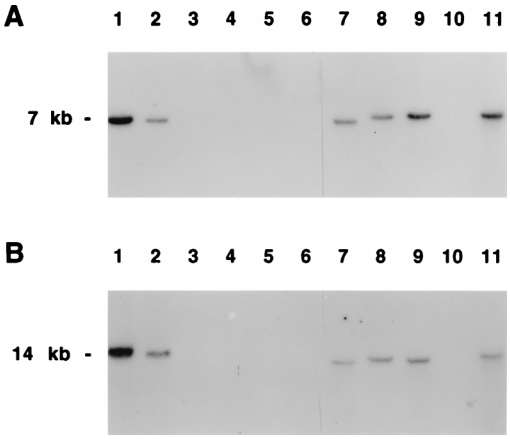

FIG. 6. Southern blot hybridization of DNAs from representative strains of *Campylobacter* probed with pGK518 (A) and a *Cla*I-*Eco*RI fragment from pRY420 which is internal to the *tonB*-like gene (B). DNAs in panel A were digested with *Cla*I, and those in panel B were digested with *Bcl*I. Lane 1, VC167 T1 (LIO8, O untypeable); lane 2, VC167 T2 (LIO8, O untypeable); lane 3, 81-176 (LIO5, O:23,36); lane 4, 81-116 (LIO6, O:6,7); lane 5, MK290 (LIO99, O:36); lane 6, MK104 (LIO4, O:19); lane 7, TGH9011 (LIO36, O:3); lane 8, MSC57360 (LIO2, O:1); lane 9, VC74 (LIO11, O:18); lane 10, MK198 (LIO not determined, O:23); lane 11, VC83 (LIO4, O not determined).

also hybridized to 27 of 33 strains of *C. jejuni* examined. The *C. jejuni* strains used were clinical isolates from either Canada, the United States, the United Kingdom, Egypt, or Thailand. All strains which hybridized to one probe also hybridized to the other. The patterns of hybridization were similar among all strains examined by Southern blotting, further suggesting that the genes are conserved among strains. Some probe-positive strains have been examined by PCR analysis using a forward primer within the *tonB* gene and a reverse primer within *cfrA*, and these data indicated that the genes are linked in all strains examined (data not shown). The six strains of *C. jejuni* examined which did not hybridize to either probe included clinical isolates from the United States, the United Kingdom, Canada, and Egypt. Among the probe-negative strains were two of the best-characterized *C. jejuni* strains: 81-176, which has been shown to utilize hemin (39), and 81116, which has been shown to utilize enterochelin (37).

DISCUSSION

Growth of *C. coli* VC167 in low-iron conditions results in induction of two major outer membrane proteins with apparent M_rs of approximately 71,000 and 75,000, as determined by SDS-polyacrylamide gel electrophoresis, similar to results reported for several strains of *C. jejuni* (14, 43). VC167 T1, like other strains, is capable of utilizing hemin, ferrichrome, and enterochelin as iron sources. The data presented here suggest that the apparent-M_r-75,000 iron-repressible protein in VC167 T1 is the product of the *cfrA* gene. Based on homology of the predicted product to known proteins and the ability of a *cfrA* mutant to utilize hemin, ferrichrome, and enterochelin, CfrA is probably a receptor for an unidentified exogenous siderophore. Pickett et al. (43) reported that a protein with an M_r of 71,000 was responsible for hemin uptake in *C. jejuni*, and the iron-repressible protein of similar M_r observed in VC167 T1 may serve the same function.

In addition, we have shown that uptake of hemin, ferrichrome, and enterochelin by *Campylobacter* is dependent on a TonB-like protein. Indeed, here we report the first *tonB* gene from any *Campylobacter* strain. Although the VC167 T1 *tonB* gene product shows very low overall homology with other TonB proteins, there are conserved motifs, and site-specific mutation of the gene results in phenotypes consistent with those of other *tonB* mutants. The *C. coli tonB* gene was unable to complement an *E. coli tonB* mutant (data not shown), but it was observed previously that the ability of *tonB* genes from heterologous bacteria to complement *E. coli tonB* mutants was directly related to the extent of identity between the *E. coli tonB* gene and the heterologous allele (28).

Interestingly, the *tonB* gene probe from VC167 T1 hybridized strongly to all strains of *C. coli* tested and numerous strains of *C. jejuni* but failed to hybridize to several other strains of *C. jejuni*. Presumably, these strains contain another *tonB* gene which is sufficiently distinct from that of VC167 T1 that it failed to hybridize, suggesting an unusually high degree of sequence diversity among campylobacter *tonB* alleles. Similarly, the absence of the *cfrA* gene in many strains of *C. jejuni* suggests either that there are two distinct alleles of *cfrA*, encoding distinct proteins which are both capable of binding to the same iron compound, or that some strains of *C. jejuni* are able to utilize iron compounds that other strains cannot use. This, in turn, may reflect a different mechanism of pathogenesis among strains and/or a difference in ecological niches that different strains can occupy.

The genetic organization of the *tonB* and *cfrA* genes in VC167 T1, with the promoters located back-to-back between the two genes, may reflect a coordinate regulatory mechanism. There are two copies of sequences resembling Fur binding boxes (9, 13, 58) in the intergenic region, but these map closer to *cfrA* than to *tonB*. The regulation of these two genes and the nature of adjacent coding sequences are currently under investigation. In addition, based on the requirement of *tonB* for virulence in *H. influenzae* (28) and iron uptake systems for virulence of *S. flexneri* (3, 48), we are also exploring the effect of mutations of *tonB* and *cfrA* on campylobacter pathogenesis in an animal model of disease (12).

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