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Molecular Characterization of a *Campylobacter jejuni* Lipoprotein with Homology to Periplasmic Siderophore-Binding Proteins

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A genomic library of *Campylobacter jejuni* (NCTC 11351) was used to identify genes which could confer a hemolytic phenotype to *Escherichia coli*. Accordingly, when transformants were screened on blood plates, hemolytic colonies appeared at a frequency of 3×10^{-4}. The gene conferring the hemolytic activity was identified by subcloning and was found to be responsible for the phenotype of all hemolytic transformants isolated. The open reading frame conferring this activity encodes a protein of 36,244 Da with a typical endopeptidase type II leader sequence. The protein is modified with palmitic acid when it is processed in *E. coli*, confirming that it is a typical lipoprotein. The deduced gene product of 329 amino acids has significant homology to the group of solute binding proteins from periplasmic-binding-protein-dependent transport systems for ferric siderophores, including the FatB protein from *Vibrio anguillarium* and the FhuD protein from *Bacillus subtilis*. In particular, the protein contained the signature sequence for siderophore-binding proteins, suggesting that the protein may be the siderophore-binding protein component of an iron acquisition system of *C. jejuni*.

Campylobacter jejuni is now recognized as the food-borne pathogen causing the most reported cases of enteritis in the United Kingdom and probably the developed world (7, 35). The disease is rarely, if ever, fatal, with symptoms ranging from a mild to a severe watery and bloody diarrhea lasting not uncommonly for 2 weeks (4, 38). The role played by several potential virulence factors in pathogenesis is unclear. These factors include enterotoxins, cytotoxins, hemolysins, and adherence factors (17, 18, 22, 29). *Campylobacter* species have been reported to be weakly hemolytic (2), and at least two different hemolysins have recently been partially characterized (15). By analogy with other species producing hemolysins, these could play a role in pathogenesis (1, 11, 16, 24). It has been suggested that some hemolysins are also involved in iron acquisition, a critical requirement for successful colonization by many enteropathogenic organisms (25). The nature of iron acquisition systems and their contribution to the virulence of *Campylobacter* species is not yet defined. For many pathogenic bacteria, the acquisition of iron, during infection, is facilitated by the production and transport of highly specific iron chelators termed siderophores (3). In gram-negative bacteria, transport of the ferric siderophore complexes is mediated by periplasmic-binding-protein-dependent systems consisting of a specific outer membrane receptor protein, a periplasmic siderophore-binding protein, a permease located in the cytoplasmic membrane, and an ATP-binding protein (3, 5). Although *C. jejuni* is known to utilize various siderophores (10), nothing is known of the mechanisms by which these ferric complexes are transported and the role that this process plays in virulence.

As a first step in characterizing potential virulence factors of *C. jejuni*, we set out to isolate the gene or genes responsible for the weakly hemolytic phenotype of this pathogen. In this study, we describe the characterization of a gene that confers a hemolytic activity upon *Escherichia coli*. The deduced gene product shows significant homology to the group of periplasmic ferric siderophore-binding proteins and contained a signature sequence representative of this group of molecules. We demonstrate that the gene encodes a lipoprotein and suggest that it is a component of a transport system for a ferric siderophore.

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MATERIALS AND METHODS

Bacterial strains and culture. The type strain NCTC 11351 of *C. jejuni* and other strains used were purchased from the National Collection of Type Cultures (Colindale, United Kingdom). *Campylobacter coli* UA585 was a gift from D. E. Taylor (University of Alberta). Campylobacter cultures were grown at 37°C on Mueller-Hinton agar under microaerophilic conditions with the recommended gas-generating kit (Oxoid Ltd., Basingstoke, United Kingdom).

DNA preparation and manipulation. Genomic DNA was isolated from *C. jejuni* by using guanidinium thiocyanate (27). A chromosomal plasmid library was created in the vector pTZ19R (23) supplied by Pharmacia. Using standard methods (21), we ligated dephosphorylated pTZ19R with *C. jejuni* chromosomal DNA which had been partially digested with *Hin*dIII. Cells were transformed with the library by electroporation (8) and plated onto Luria-Bertani agar containing ampicillin (50 μg/ml) and 5% sheep blood (Oxoid Ltd.). Transformants were grown at 37°C for 48 h, after which zones of hemolysis in some colonies were observed. The DNA from the hemolytic clones was prepared after amplification by standard methods prior to insert mapping and sequence analysis (21). Only two types of insert were obtained: a 10-kb fragment and a 6-kb fragment. These fragments were sequenced by standard methods (30) with a Sequenase version 2.0 kit (U.S. Biochemicals). The genes from both the 10- and 6-kb fragments were sequenced entirely with primers designed to bind to opposing strands at approximately 200-bp intervals to allow sequence data to be obtained from both strands separately. Oligonucleotides were synthesized with a model 391 DNA synthesizer (Applied Biosystems). Computer analysis of the sequence data was carried out by using the University of Wisconsin molecular biology software package (6).

Analysis of plasmid-encoded proteins. The polypeptides encoded by pCJH3-A and pCJH3-D were identified with an *E. coli* in vitro coupled transcription/translation system (Promega). Proteins were radiolabelled by incubation with 20 μCi of [^{35}S]methionine (Amersham). Polypeptides were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels (20) with Rainbow ^{14}C-methylated markers (Amersham) as molecular size standards.

Lipoproteins were labelled specifically with [^{3}H]palmitic acid. Cells containing the various plasmids were grown in Luria-Bertani broth, and protein expression was induced with 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells growing exponentially were incubated in the presence of 20 μCi of [9,10 (*n*)-^{3}H]palmitic acid (Amersham) ml^{-1} at 37°C for 4 h. Cells were harvested and were washed twice in methanol to remove unincorporated radioactive precursors

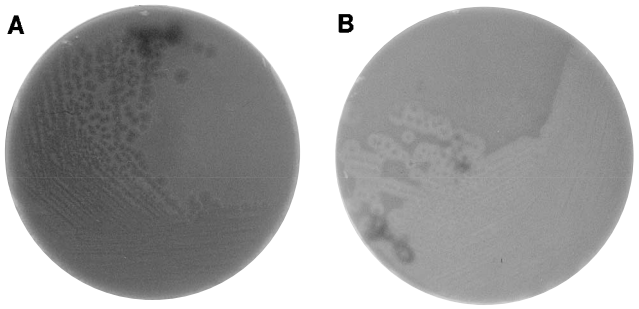

FIG. 1. Hemolysis of *E. coli* transformed with plasmids containing *C. jejuni* genomic DNA on sheep blood agar plates. Each clone was plated on Luria-Bertani agar plates containing 50 μg of ampicillin ml^{-1} and 5% sheep blood and grown for 48 h at 37°C. (A) pTZ-transformed culture (vector alone). (B) pCJH3-transformed culture.

and noncovalently bound lipids. The dried cell pellets were then boiled in SDS-loading buffer and analyzed on SDS-polyacrylamide gels as described above. Radiolabelled bands were visualized by fluorography at −70°C.

DNA-DNA hybridizations. DNA-DNA hybridizations were carried out with a nonradioactive enhanced chemiluminescence gene detection kit (Amersham). Chromosomal DNAs from various strains of *C. jejuni* and one strain of *C. coli* (UA585) were restricted with *Hin*dIII and electrophoresed on a 0.8% agarose gel. Gel-electrophoresed DNA was depurinated by gently shaking the gel in 250 mM HCl for 30 to 45 min, and the DNA was transferred to Hybond N+ nitrocellulose membranes (Amersham) by an alkali transfer procedure (21). The DNA insert in pCJH3-D was labelled and used as a DNA probe to detect cross-hybridizing species in accordance with the manufacturer's guidelines.

Nucleotide sequence accession number. The GenBank accession number for the sequence presented in this article is X82427.

RESULTS

Cloning and localization of the gene conferring hemolytic activity. A genomic library of DNA from *C. jejuni* NCTC 11351, which consisted of approximately 10,000 independent clones containing inserts of between 4 and 7 kb, was constructed in *E. coli* SURE. From this population, three transformants exhibiting distinct zones of hemolysis were isolated when clones containing the library were screened on blood agar plates. The expression of the hemolytic phenotype on blood agar is compared with that of the vector-containing strain in Fig. 1. Plasmid DNA purified from one hemolytic transformant was shown to contain 6.0 kb of *C. jejuni* DNA and was designated pCJH3. To aid further molecular analysis, several deletion subclones were constructed in order to localize the gene conferring the hemolytic phenotype (Fig. 2). Initially, the gene encoding the hemolytic phenotype was found to reside in a 3,167-bp insert generated by digestion of pCJH3 with

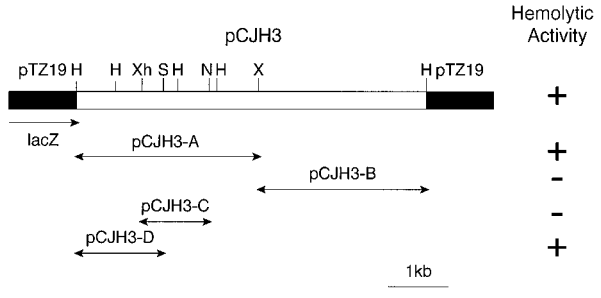

FIG. 2. Strategy for subcloning of the gene from *C. jejuni* which confers hemolytic activity. Subclones of pCJH3 are indicated (arrowed lines) below their restriction sites in the parental clone. The hemolytic phenotype (on blood agar) of *E. coli* transformed with each particular subclone is indicated. H, *Hin*dIII; N, *Nru*I; S, *Ssp*I; X, *Xba*I; Xh, *Xho*I.

*Xba*I (pCJH3-A in Fig. 2). The gene encoding the activity was then assigned to a 1,581-bp fragment by digestion of pCJH3-A with *Ssp*I (pCJH3-D in Fig. 2). The other two hemolytic transformants were both shown to contain plasmids, designated pCJH1 and pCJH2, which harbored identical 10-kb inserts. Analysis of these plasmids has shown them to contain the same open reading frame responsible for generating the hemolytic activity in pCJH3 and pCJH3-D (data not shown).

Sequence analysis of the gene encoding a hemolytic phenotype. The nucleotide sequence of the 1,581-bp insert in pCJH3-D that conferred the hemolytic phenotype was determined (Fig. 3). The DNA fragment contained a single open reading frame which started with an ATG at base 133 and ended with a TAA at base 1020. The G+C content of the open reading frame is 31%, which agrees closely with that of 32.5% predicted for the *C. jejuni* genome. The sequence AAGGAG which is a possible ribosome binding site (12) is located 5 bp upstream of the translational start codon. At bases 61 to 66, the sequence TATgAT is conserved (written in uppercase letters) in five of the six nucleotides of the −10 region for a promoter element. Upstream of this region at bases 39 to 44 is the sequence TcaAgA which matches two of the six nucleotides in the −35 region of a promoter structure (14). In a variety of other bacterial species, iron transport systems are regulated by the Fur repressor protein which binds to a consensus sequence (3). In the vicinity of the −10 region of the putative promoter region, at bases 64 to 82, is the sequence GATAtgGATAtac ccgta which is conserved in 8 of the 19 bases (written in uppercase letters) of the Fur consensus sequence (3). Downstream of the termination codon of the open reading frame, between bases 1324 and 1342, is an inverted repeat typical of a rho-independent termination signal. The open reading frame encodes a protein of 329 amino acids with a calculated molecular mass of approximately 36.2 kDa. The 21 amino acids representing the N terminus of the protein have the characteristics of a lipoprotein signal sequence (28, 37). In particular, this region contains the sequence LTAC which matches the consensus sequence thought to be recognized by signal peptidase II (28, 37).

A search of the GenBank database, using the derived amino acid sequence, revealed no significant homology with any protein previously associated with hemolytic activity. Instead, the search revealed that the protein had marked homology over all 329 amino acids with a group of ferric siderophore-binding proteins (Fig. 4). The protein showed the highest degree of homology (29.9% identity) with FatB, the periplasmic binding protein for angubactin from *Vibrio anguillarium* (19). Lower-percentage but still significant homologies were also apparent with FhuD (28.5% identity), the iron hydroxamate-binding protein from *Bacillus subtilis* (31); FecB (25.2% identity), the binding protein for ferric citrate in *E. coli* (33); and FepB (24.4% identity), the binding protein for enterochelin in *E. coli* (9). Furthermore, a region of amino acids from position 137 to 152 (Fig. 4, sequence QVDF**E**AINALK**P**DLII, with the invariably conserved residues in boldface type) shows strong conservation in comparison with an amino acid sequence which has been identified as a signature region for periplasmic siderophore-binding proteins (34).

Analysis of proteins specified by pCJH3-A and pCJH3-D in an in vitro transcription/translation system. The proteins specified by pCJH3-A and pCJH3-D are indicated on the gel in Fig. 5A. Both plasmids produce a 36-kDa polypeptide which is not produced from the parental vector alone (pTZ19R). The plasmid pCJH3-D contains only one open reading frame within the cloned *C. jejuni* DNA, encoding a polypeptide of approximately 36 kDa. It is likely, therefore, that the 36-kDa

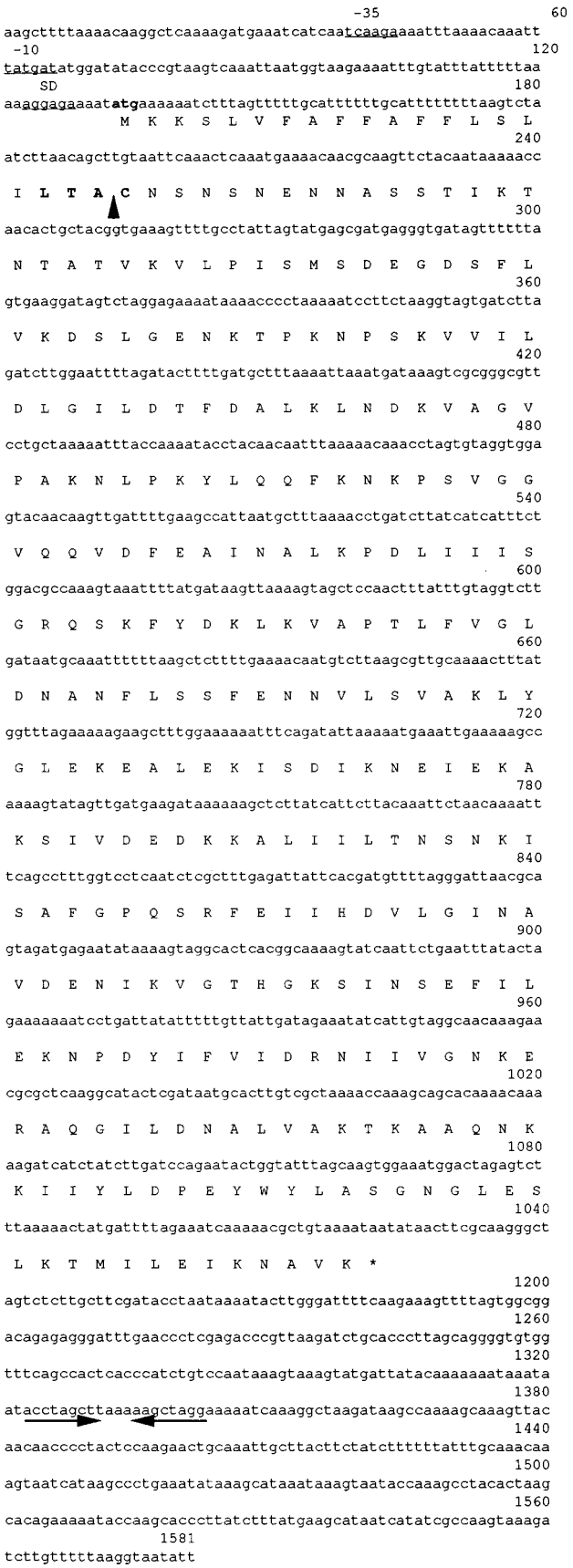

FIG. 3. Nucleotide and amino acid sequences of the 1,581-bp insert of *C. jejuni* DNA in pCJH3-D. The putative ribosome binding site (SD) and possible promoter sequences (−10 and −35) are underlined. The amino acid sequence which conforms to the consensus sequence recognized by signal peptidase II is shown in boldface type and the predicted cleavage site is marked by a vertical arrow. A dyad repeat indicative of a termination signal is indicated by horizontal arrows. Nucleotide positions are numbered on the right.

protein produced in the expression system is the product of this gene and represents the protein with homology to siderophore-binding proteins. In addition, two additional polypeptides of 23 and 25 kDa are produced by plasmid pCJH3-A. This construct

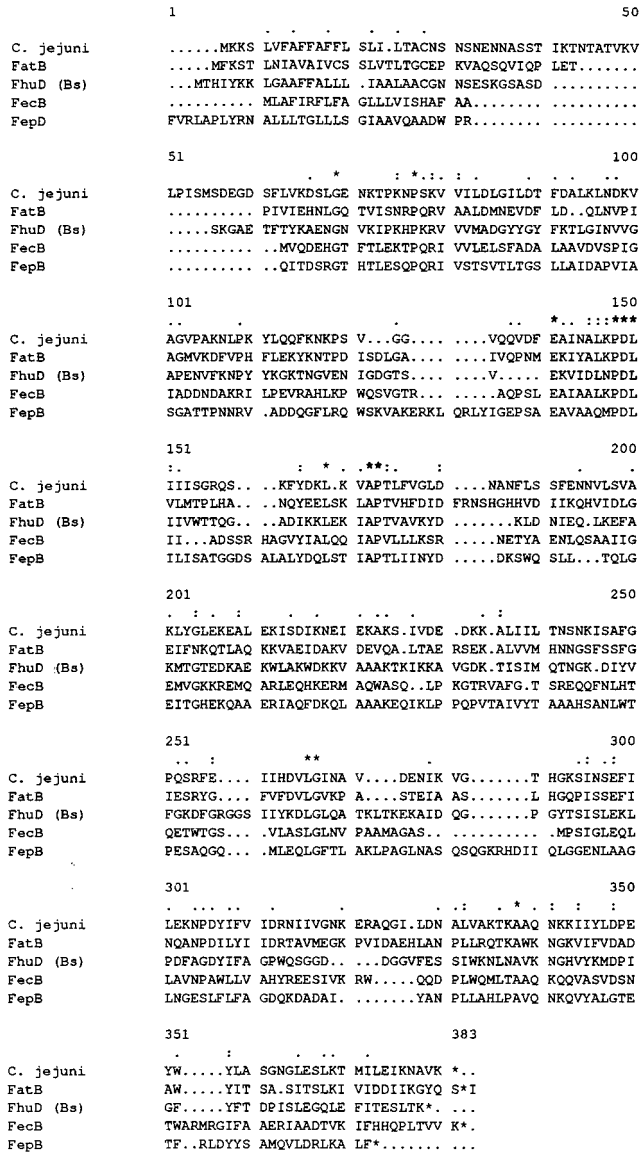

FIG. 4. Amino acid similarities between the *C. jejuni* gene product which confers a hemolytic activity and siderophore-binding proteins from other species. FatB is derived from *V. anguillarium*, FhuD is derived from *B. subtilis* (Bs), and FecB and FepD are derived from *E. coli*. Three identical amino acids in a position (as numbered) are denoted by a period, four are indicated by a colon, and five are shown by an asterisk above the sequences. Periods and asterisks within individual sequences indicate sequence gaps and termination codons, respectively.

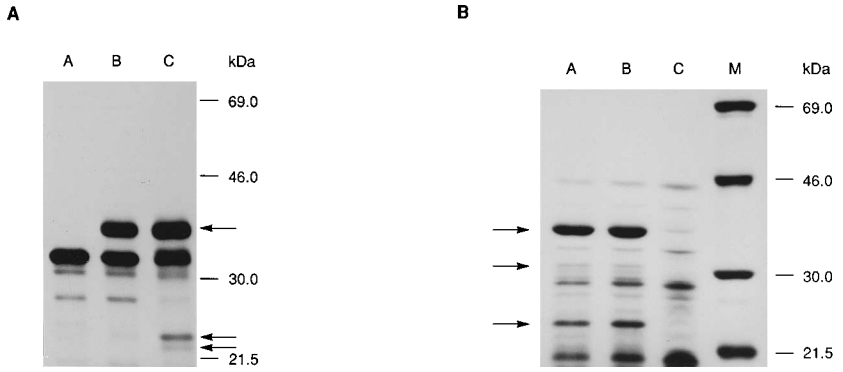

FIG. 5. Expression and lipophilic modification of the protein with homology to siderophore-binding proteins. (A) Fluorograph of [^{35}S]methionine-labelled proteins expressed in an in vitro-coupled transcription/translation system using plasmids pTZ19 (lane A), pCJH3-A (lane C), and pCJH3-D (lane B) as templates. (B) Fluorograph from cells containing the plasmids pTZ19 (lane C), pCJH3-A (lane B), and pCJH3-D (lane A) grown in the presence of [^{3}H]palmitate, with the labelled proteins analyzed by SDS-polyacrylamide gel electrophoresis. The arrows designate proteins that are produced uniquely from specific plasmids. The positions and sizes of standard proteins (lane M) are indicated.

contains an extra 1,555 bp of the *C. jejuni* chromosome, containing additional open reading frames (as yet unidentified) which most likely specify these products. Expression of the 36-kDa protein and the hemolytic phenotype was independent of the orientation of the DNA insert within pTZ19, which suggests that the *C. jejuni*-derived transcriptional control sequences were fully functional in *E. coli*. Indeed, removal of 132 bp upstream of the ATG start by PCR led to the abolition of expression of the hemolytic phenotype (data not shown).

Posttranslational modification of the protein homologous to siderophore-binding proteins. The polypeptide with homology to siderophore-binding proteins has an N-terminal signal sequence which contains a consensus sequence for signal endopeptidase II (37), suggesting that the protein may be subject to lipophilic modification. To confirm the nature of the putative lipoprotein, *E. coli* cells containing recombinant plasmids pCJH3-A and pCJH3-D were grown in the presence of [^{3}H]palmitic acid to effect the specific labelling of lipoproteins. As shown in Fig. 5B, a lipoprotein of 33.8 kDa is produced by cells containing pCJH3-A and pCJH3-D but not by cells containing just the parental vector, pTZ19. The decrease in size of the polypeptide, compared with that of the product (36 kDa) obtained in the in vitro expression system, is consistent with cleavage of the signal sequence following posttranslational modification by endopeptidase II. Two smaller additional lipophilically labelled polypeptides of 31 and 25 kDa are also produced uniquely by cells containing pCJH3-A and pCJH3-D and probably represent degradation products of the 33.8-kDa protein.

Southern hybridization analyses. To confirm that the open reading frame encoding the siderophore-binding protein originated from *C. jejuni* and to assess its distribution amongst other strains and species, Southern hybridization analyses using *Hin*dIII-digested chromosomal DNA from various *C. jejuni* strains and one strain of *C. coli*, with the cloned DNA from pCJH3-D as a DNA probe, were performed. As shown by Fig. 6, the probe was found to hybridize to 0.67- and 1.1-kb fragments from the parental *C. jejuni* strain (NCTC 11351). This pattern was expected, as the open reading frame contains an internal *Hin*dIII site, and confirms that the gene is present as a single copy and is of *C. jejuni* origin. In addition, *C. jejuni* NCTC 11322 and NCTC 11168 strains show an identical pattern of hybridization, suggesting that these strains contain an equivalent gene with a similar organization. In contrast, strains *C. jejuni* 81116, NCTC 11392, and NCTC 11951 hybridized to the probe but with a different pattern. In these strains, the probe hybridized not only to the common 0.67-kb fragment but also to fragments of differing sizes: 2.0 kb for strains 81116 and NCTC 11392 and 2.5 kb for NCTC 11951. This result indicates that a homologous gene is also present in these strains but that the arrangement of *Hin*dIII sites is different. Sequence analysis of the *C. jejuni* strains showing these alternative hybridization patterns has confirmed the presence of identical genes (data not shown). The *C. coli* UA 585 strain also hybridized with the probe, albeit with a unique pattern, suggesting that a homolog of this gene also exists in this species.

DISCUSSION

There is increasing evidence that strains of *C. jejuni* produce one or more hemolysins and that these may be involved in virulence (2, 15, 26). The genetic characterization of a number of hemolysins from other bacterial pathogens has been facilitated greatly through the use of recombinant DNA techniques. For instance, the gene encoding a hemolysin from *Listeria monocytogenes* has been cloned because it confers a hemolytic phenotype on *E. coli* (36). We have cloned a genetic determinant from *C. jejuni* NCTC 11351 which confers a hemolytic phenotype when it is expressed in *E. coli*. Initially, three hemolytic clones were isolated when transformants containing a

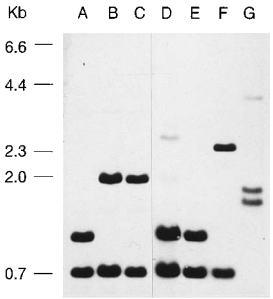

FIG. 6. Distribution of genes encoding the protein with homology to siderophore-binding proteins in *C. jejuni* and *C. coli*. The inserted DNA in pCJH3-D was labelled and used to probe genomic DNAs from *C. jejuni* NCTC 11351 (lane A), 81116 (lane B), NCTC 11392 (lane C), NCTC 11322 (lane D), NCTC 11168 (lane E), and NCTC 11951 (lane F) and *C. coli* UA585 (lane G) which had been digested with *Hin*dIII. The sizes and positions of molecular standards are shown on the left.

library of *C. jejuni* DNA were screened on blood agar. Analysis of the plasmid DNA contained within these clones showed that either a 6.0- or a 10.0-kb insert was responsible for the hemolytic phenotype. However, further analysis revealed that the inserts overlapped and contained the same open reading frame. The calculated pI of this gene product (8.3) differs from that of previously characterized hemolysins (15), indicating that this is a different molecule.

The gene responsible for the hemolytic phenotype was located by subcloning and shown to encode a polypeptide of 329 amino acids. An amino acid sequence homology search of GenBank did not reveal significant homology to previously characterized hemolysins. However, the protein showed significant homology to a group of periplasmic binding proteins, which are components of transport systems for ferric siderophores, from gram-negative bacteria (9, 33) and to the ferrichrome receptor FhuD of *B. subtilis* (31). In gram-negative bacteria, ferric siderophore transport involves several proteins, including an outer membrane receptor, a soluble periplasmic binding protein, and two or more proteins associated with the cytoplasmic membrane (3, 5). The protein from *C. jejuni* that confers the hemolytic phenotype on *E. coli* showed significant homology to the periplasmic-binding-protein components of these ferric transport systems and, furthermore, contained a signature sequence characteristic of these molecules (34). It is likely, therefore, that this protein is a component of a periplasmic-binding-protein-dependent system for ferric siderophores and not a true hemolysin. Indeed, although the expression of the siderophore-binding protein clearly conveys a hemolytic phenotype when cells are grown on plates (Fig. 1), we have been unable to detect any hemolytic activity in culture supernatants or in total-cell lysates. It is, therefore, probable that the hemolysis observed on plates is an indirect effect caused by lysis of the *E. coli* host by the overexpression of a protein which may have a toxic nature. In this respect, we have shown that the protein responsible for the hemolytic phenotype is lipophilically modified when it is expressed in *E. coli*. It is likely, therefore, that the mechanism of cell lysis is similar to that previously attributed to the expression of a lipoprotein from *B. subtilis*, which was responsible for conferring a falsely α-amylytic phenotype on the *E. coli* host (13).

Periplasmic-binding-protein-dependent transport systems also exist in gram-positive bacteria (31). However, since this group of bacteria have no outer membranes, there is no requirement for outer membrane receptor proteins. Instead, the periplasmic receptor proteins are lipoproteins thought to be anchored to the cytoplasmic membrane to prevent their escape into the environment (31). The gene encoding the protein with homology to siderophore-binding proteins identified in this study encodes a protein having a signal sequence which shows all the characteristics of a lipoprotein signal sequence (28, 37), i.e., containing the sequence LTAC which conforms to the consensus sequence recognized by signal peptidase II (37). These amino acid sequences are covalently modified at the cysteine residue and cleaved by signal peptidase II to generate the mature lipoproteins. The presence of this type of signal sequence, therefore, strongly suggests that the protein is a lipoprotein. Indeed, we have demonstrated that the protein is modified with [^{3}H]palmitate when it is expressed in *E. coli*, confirming that it is a typical lipoprotein. If this protein is the periplasmic-binding-protein component of a transport system for ferric siderophores, which seems very likely, then it would be unusual since in all of the previously characterized ferric-siderophore transport systems from gram-negative bacteria the periplasmic-binding-protein component is not a lipoprotein (9, 19, 33). In contrast, the recently characterized FhuD protein from *B. subtilis*, which is the binding-protein component of the transport system for iron(III) hydroxamate, is a lipoprotein (31). In addition, a recently identified protein which may serve as a receptor for a ferric siderophore complex in another gram-positive bacterium, *Corynebacterium diphtheriae*, is also thought to be a lipoprotein (32). Although *C. jejuni* is considered gram negative, it would seem that if the protein from *C. jejuni* is the periplasmic-binding-protein component of a siderophore transport system, then as a lipoprotein it has structural features in common with analogous components from solute transport systems in gram-positive bacteria. The reasons for this dual nature are unclear at present, but it may reflect either the origin of the transport system or differences between the physiology of *C. jejuni* and that of other gram-negative bacteria.

Elucidation of the mechanisms of iron acquisition in *C. jejuni* should aid the understanding of both the physiology and pathogenesis of this bacterium. The description of a lipoprotein with homology to siderophore-binding proteins may represent an important first step towards this characterization.

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