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Molecular Characterization of a *Campylobacter jejuni* 29-Kilodalton Periplasmic Binding Protein

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***Campylobacter jejuni*, a gram-negative, microaerophilic, spiral bacterium, is a common cause of human gastrointestinal disease. Although investigators commonly use *C. jejuni* glycine-hydrochloride extracts in assays to determine the products that promote the binding of the organism to eukaryotic cells, the proteins contained within these extracts remain ill defined. Characterization of these proteins will provide a better understanding of *C. jejuni* gene regulation and organization. An antiserum was raised against a *C. jejuni* 29-kDa gel-purified protein detected in glycine-hydrochloride extracts. This antiserum was used to screen an expression library of *C. jejuni*. A reactive clone that contained an open reading frame of 256 amino acids was identified. The cloned gene was transcribed and translated, and the product was exported to the periplasmic space in *Escherichia coli* XL1-Blue. The translated *C. jejuni* product, designated P29, exhibited significant similarity to the histidine and lysine-arginine-ornithine periplasmic binding proteins (HisJ and LAO, respectively) of *Salmonella typhimurium*. The *C. jejuni* gene encoding the P29 protein complemented an *S. typhimurium* HisJ mutant but not a LAO mutant when provided in *trans*. These data suggest that the *C. jejuni* gene encoding the P29 protein is a homolog of the *S. typhimurium hisJ* gene.**

Campylobacter jejuni is frequently isolated from individuals suffering from acute gastrointestinal disease (2). Infection with *C. jejuni* is often acquired by ingestion of undercooked chicken, unpasteurized milk, or untreated water. *C. jejuni* infection is characterized by the sudden onset of fever, abdominal cramps, and diarrhea with blood and leukocytes. Despite the recognition of *C. jejuni* as a common cause of gastrointestinal disease, the pathogenic mechanisms associated with *C. jejuni* gastrointestinal disease are ill defined. Potential virulence determinants include cytotoxins and the products that mediate the binding of the organism to (3, 7, 11, 13, 23) and entry into (12, 13, 17, 21) eukaryotic cells (19, 32).

A number of investigators have used outer membrane protein and glycine-hydrochloride extracts in ligand-binding assays to identify the bacterial products that promote the binding of *C. jejuni* to eukaryotic cells (4, 6). Products proposed to facilitate binding range in molecular mass from 26 to 30 kDa (6). Although the treatment of *C. jejuni* with glycine-hydrochloride is thought to result in the extraction of cell surface and periplasmic proteins (18), the components of the glycine-hydrochloride extracts remain only partially characterized.

To define one component of *C. jejuni* glycine-hydrochloride extracts, a rabbit antiserum was raised against a protein with an apparent molecular mass of 29 kDa detected in the extracts. This rabbit anti-29-kDa serum was then used to screen a *C. jejuni* expression library. A *C. jejuni* gene that encodes a protein of 256 amino acids with a calculated molecular mass of 28,531 Da (P29) was cloned. The deduced amino acid sequence of this gene exhibited homology with the histidine (HisJ) and lysine-arginine-ornithine (LAO) periplasmic binding proteins from *Salmonella typhimurium*. The cloned *C. jejuni* gene complemented an *S. typhimurium* HisJ mutant when provided in *trans*, suggesting that it encodes a histidine periplasmic binding protein homologous to the *S. typhimurium* HisJ product. Binding assays showed that *Escherichia coli* transformants expressing the cloned *C. jejuni* gene did not adhere to INT407 cells in greater numbers than did transformants harboring the parental, pBluescriptII SK$^+$ (pBSKII$^+$) plasmid.

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MATERIALS AND METHODS

Bacterial isolates and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* clinical isolate M275 was cultured at 37°C on Mueller-Hinton agar plates containing 5% citrated blood in Gas-Pak jars with CampyPak Plus packets (BBL Microbiology Systems, Becton Dickinson, Cockeysville, Md.). This isolate was passaged every 24 to 48 h.

E. coli XL1-Blue and *S. typhimurium* periplasmic binding mutants were subcultured on Luria-Bertani (LB) agar plates (10 g of Bacto-Tryptone, 5 g of yeast extract, 10 g of sodium chloride, and 15 g Bacto-Agar per liter) in a 37°C incubator. The plates were supplemented with ampicillin (50 μg/ml; Boehringer Mannheim, Indianapolis, Ind.) and kanamycin (50 μg/ml; Boehringer Mannheim) as appropriate. The *S. typhimurium* mutants TA3286 and TA3296 were provided by G. F.-L. Ames (15).

Electrophoretic and immunoblot analysis. Bacterial whole-cell, glycine-hydrochloride, and osmotic shock samples were solubilized in single-strength electrophoresis sample buffer (16) and heated to 95°C for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) (SDS-PAGE) with the discontinuous buffer system described by Laemmli (16). The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore) with the buffer system described by Towbin et al. (29). The membranes were incubated for 18 h at 4°C with a 1:250 dilution of rabbit anti-*C. jejuni* 29-kDa serum in phosphate-buffered saline (PBS; pH 7.4)–0.01% Tween 20 containing 20% bovine serum and an *E. coli* extract (Protoblot; Promega, Madison, Wis.) to reduce background. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika Corp., West Chester, Pa.) and 4-chloro-1-naphthol (Sigma, St. Louis, Mo.) as the chromogenic substrate. For gel electrophoresis, 20 μg of protein was loaded per lane. Protein concentrations were determined by the bicinchoninic assay (Pierce Laboratories, Rockford, Ill.) with bovine serum albumin as the standard (27).

Glycine-acid extraction. Mild glycine-acid treatment of cells, with minimal disruption, is commonly used for extraction of *C. jejuni* membrane-associated proteins (4, 5, 24). *C. jejuni* cells were pelleted by centrifugation at 6,000 × *g* for 10 min at 4°C, washed twice with 10 mM phosphate buffer (pH 7.0), suspended in 0.2 M glycine-hydrochloride (pH 2.2), and stirred for 30 min at room temperature. Bacterial cells were removed by centrifugation at 6,000 × *g* for 20 min, and the supernatant fluid was neutralized with NaOH. The glycine-acid-extracted material was dialyzed against PBS, concentrated with a Centricon-10 (Amicon, Inc., Beverly, Mass.), and stored frozen at −20°C.

Osmotic shock. Periplasmic binding proteins from *C. jejuni* and *E. coli* XL1-Blue harboring either the recombinant or parental (pBSKII+) plasmids were isolated by the osmotic shock procedure (1). Briefly, 0.5 M Tris-Cl (pH 7.8) was

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli XL1-Blue	Host for propagation and maintainence of all plasmids	Stratagene
C. jejuni M275	Clinical isolate; source of genomic DNA	14
S. typhimurium		
TA3286	*gln-139 argT526 dhuA1 hisF645*	15
TA3296	*gln-139 dhuA1 hisJ5601 hisF645*	15
Plasmids		
pBluescriptII SK$^+$	Ap^r; *lacZ′* with multiple cloning sites	Stratagene
pMEK45	Ap^r; ~2.2-kb *Bgl*II chromosomal DNA fragment in *Bam*HI site of pBluescriptII SK$^+$	This study
pMEK45-8	Ap^r; 1.1-kb *Hin*dIII chromosomal fragment in *Hin*dIII site of BluescriptII SK$^+$ (the *Cla*I site in the vector was removed prior to insertion of *Hin*dIII fragment)	This study
pMEK45-8::*kan*	Ap^r Km^r; 1.4-kb fragment containing *kan* gene cloned into the *Cla*I site of the 1.1-kb chromosomal DNA fragment	This study

[a] Abbreviations: Ap^r, resistance to ampicillin; Km^r, resistance to kanamycin.

added to a final concentration of 50 mM to an overnight culture of bacterial cells. The bacteria were pelleted by centrifugation and suspended in 50 mM Tris-Cl containing 40% sucrose to 1/10 of the original culture volume. Immediately after suspension of the cell pellet, 0.5 M EDTA was added to a final concentration of 2 mM. The cells were gently stirred for 10 min at room temperature and pelleted by centrifugation. The bacterial pellet was then suspended in cold distilled water to 1/10 of the original culture volume and pelleted by centrifugation. The supernatant fluid, containing the periplasmic binding proteins, was harvested and concentrated with a Centricon-10. Osmotic shock fluids were stored frozen at −20°C.

Preparation of antiserum. Antiserum was raised in a 2-month-old New Zealand White rabbit by methods described previously (9, 28). *C. jejuni* M275 glycine-hydrochloride extracts were separated by SDS-PAGE and stained with Coomassie brilliant blue R250. After destaining, a protein with an apparent molecular mass of 29 kDa was excised from the gel. The excised gel slices were suspended in 1 ml of sterile saline, homogenized with a syringe fitted with a 22-gauge needle, and injected into the lumen of a polyethylene chamber surgically implanted in the rabbit. A booster injection was given after 4 weeks. Serum was collected from the polyethylene chamber before the first and second immunizations and 2 weeks after the second immunization. An average of 10 ml of fluid was collected each time from the lumen of the polyethylene chamber. The serum was stored at −20°C.

Construction and screening of *C. jejuni* genomic libraries. *C. jejuni* genomic DNAs were isolated as described previously (20). Partially *Bgl*II-digested *C. jejuni* M275 chromosomal DNA was ligated into the pBSKII$^+$ plasmid, which had been digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (Promega) with T4 DNA ligase (Boehringer Mannheim) in an overnight incubation at 14°C under the conditions described by the supplier. After the addition of 5 μg of herring sperm DNA, the ligated products were precipitated by the addition of 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol. The precipitated DNA was washed once with 70% ethanol, air dried, and suspended in water. The DNA was then transformed into *E. coli* XL1-Blue by electroporation with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.).

Screening of the *C. jejuni* genomic library. Transformed *E. coli* XL1-Blue cells were plated onto LB agar containing 50 μg of ampicillin per ml and incubated for 18 h at 37°C. The bacterial colonies were transferred to Duralose-UV membranes (Stratagene) and incubated for 15 min on a piece of 3MM paper (Whatman) saturated in chloroform. After incubation, the membranes were allowed to dry and incubated with a 1:250 dilution of the rabbit anti-*C. jejuni* 29-kDa serum in PBS (pH 7.4) containing 0.01% Tween 20 and 20% bovine serum. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G and 4-chloro-1-naphthol as the chromogenic substrate. Colonies giving a positive signal were isolated and cultured in LB broth containing 50 μg of ampicillin per ml. Plasmid DNA was purified on an ethidium-CsCl density gradient after preparation of a cleared lysate as described by Sambrook et al. (26).

DNA sequencing. DNA sequencing was performed with a double-stranded DNA cycle-sequencing kit as indicated by the supplier (Life Technologies Inc., Gaithersburg, Md.). Primers were synthesized by Ransom Hill Bioscience, Inc., Ramona, Calif. Samples were heated to 95°C for 5 min prior to electrophoresis in 8% polyacrylamide–8 M urea sequencing gels in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA [pH 8.0]). After electrophoresis, the gels were transferred to 3MM paper, dried, and analyzed by autoradiography.

Primer extension. Bacterial RNA from *C. jejuni* M275 was isolated as previously described (20). All reagents and glassware were prepared or treated with diethylpyrocarbonate-treated water by standard methods. The transcriptional start site of the gene encoding the P29 protein was determined by using an avian myeloblastosis virus reverse transcriptase primer extension system as outlined by the supplier (Promega). The radiolabeled primer extension (RNA-resistant cDNA) product was treated with DNase-free RNase (Boehringer Mannheim), precipitated with 100% ethanol, washed with 70% ethanol, vacuum dried, and suspended in 20 μl of formamide loading buffer. The sample was heated at 95°C for 5 min and electrophoresed in an 0.8% polyacrylamide–8 M urea sequencing gel in TBE buffer. A DNA ladder was used to determine the size of the primer extension product. The gels were transferred to 3MM paper (Whatman) immediately after electrophoresis and analyzed by autoradiography.

Southern hybridization. Southern hybridization of *C. jejuni* M275 chromosomal DNA with the nick-translated pMEK45 plasmid was performed under conditions described elsewhere (20). Plasmid DNA was nick translated with [α-^{32}P]dCTP (New England Nuclear, Boston, Mass.), using a nick translation kit from Promega Corp. *C. jejuni* chromosomal DNA was digested with restriction endonucleases under the conditions described by the supplier (New England Biolabs, Beverly, Mass.), fractionated in 0.8% agarose gels (16 h at 25 V) in TBE buffer, and transferred to GeneScreen hybridization transfer membranes (New England Nuclear) by vacuum blotting with a VacuGene vacuum blotting apparatus as recommended by the manufacturer (Pharmacia). The DNA was fixed to the membrane by UV cross-linking with a GS gene linker UV chamber (Bio-Rad).

In vitro transcription-translation analysis. In vitro transcription-translation analysis was performed to identify the products of the pMEK45 recombinant plasmid by using an *E. coli* S30 extract system for circular DNA as outlined by the manufacturer (Promega). Proteins produced from the plasmid (4 μg per reaction) were radiolabeled with L-[^{35}S]methionine (New England Nuclear).

Complementation experiments. Plasmids pMEK45-8 and pMEK45-8::*kan* were introduced into the *S. typhimurium* mutants TA3286 and TA3296 by electroporation, using standard methods. Plasmid carriage was confirmed by performing plasmid DNA isolations and restriction enzyme analyses. The production of the P29 protein was confirmed by immunoblotting with the rabbit anti-*C. jejuni* 29-kDa serum. For complementation analyses, bacterial strains were grown overnight in LB broth containing 50 μg of ampicillin per ml, washed twice in minimal M-9 medium, and suspended in minimal M-9 medium. Each bacterial strain (0.001 optical density [600 nm] units) was then inoculated into 15 ml of minimal M-9 medium supplemented with 0.4% glucose, 0.001% thiamine, 10 mM ammonium chloride, 50 μg of ampicillin per ml, and the appropriate amino acid. *S. typhimurium* TA3296 transformants harboring pMEK45-8 or pMEK45-8::*kan* were cultured in supplemented M-9 minimal medium containing 0.1 mM D-histidine, and *S. typhimurium* TA3286 transformants harboring pMEK45-8 or pMEK45-8::*kan* were cultured in supplemented M-9 minimal medium containing both 0.1 mM D-histidine and 0.1 mM L-arginine. The bacteria were cultured in 50-ml conical tubes with shaking (225 rpm) at 37°C. Growth was monitored turbidimetrically by measuring the optical density at 600 nm.

Adherence assays. Adherence assays were performed with *E. coli* transformants harboring pBSKII$^+$ or pMEK45 as described elsewhere (11). Briefly, INT 407 (human embryonic intestine; ATCC CCL 6) stock cultures were grown as monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum in 75-cm^2 flasks at 37°C in a humidified, 5% CO_2 incubator. For adherence assays, each well of a 24-well tissue culture tray (Costar, Cambridge, Mass.) was seeded with 7.5×10^4 cells and incubated for 18 h under the conditions described above. The semiconfluent monolayers were then rinsed once with Eagle's minimal essential medium supplemented with 1% fetal bovine serum, inoculated with 5×10^7 CFU of a bacterial suspension, centrifuged at $600 \times g$ for 5 min, and incubated at 37°C in a humidified, 5% CO_2 incubator. After incubation, the monolayers were rinsed three times with PBS and the epithelial cells were lysed with a solution of 0.5% (wt/vol) sodium deoxycholate. The suspensions were diluted, and the number of viable, adherent

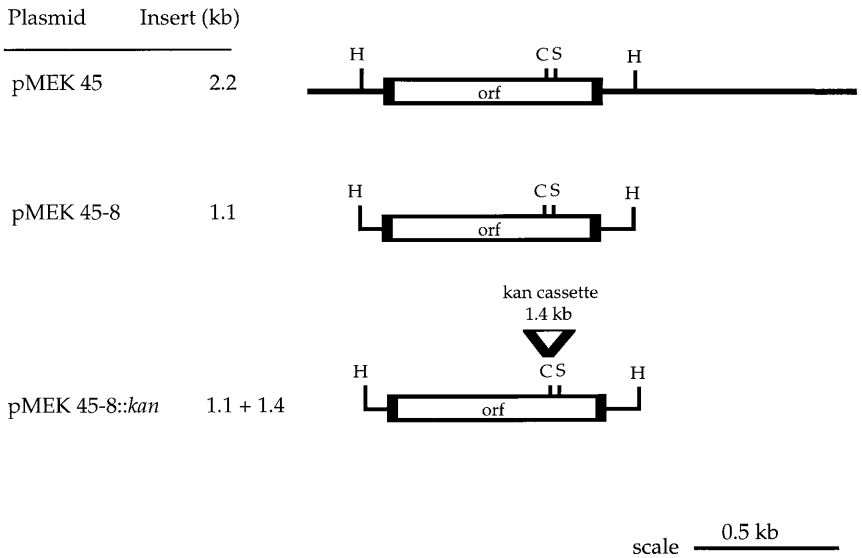

FIG. 1. Physical maps of pMEK45 and its derivatives. The top line shows a partial restriction map of the cloned 2.2-kb *C. jejuni* insert contained within the pMEK45 recombinant plasmid. Not shown in pMEK45 are the *Hin*dIII and *Sau*3AI restriction enzyme sites flanking the internal 1.2-kb *Hin*dIII fragment. Line 2 is the partial restriction map of pMEK45-8. Line 3 shows the pMEK45::*kan* insert, in which a 1.4-kb fragment containing the *kan* gene was ligated in the *Cla*I site of pMEK45-8. The open boxes indicate the region of the ORF within the cloned inserts. Sites: H, *Hin*dIII; C, *Cla*I; S, *Sau*3AI. The plasmid designation and size of the each insert, in kilobases, are indicated on the right.

bacteria was determined by counting the resultant colonies on Mueller-Hinton agar plates containing 5% citrated blood. Positive and negative controls consisted of *C. jejuni* M275 and *E. coli* transformants harboring the pBSKII$^+$ parental plasmid, respectively.

Nucleotide sequence accession number. The GenBank accession number of the sequence shown in Fig. 2 is U58045.

RESULTS

Isolation of an *E. coli* transformant expressing a 29-kDa recombinant product. To characterize a *C. jejuni* component extractable by glycine-hydrochloride treatment, an antiserum was raised against a gel-purified protein with an apparent molecular mass of 29 kDa. The rabbit anti-P29 serum was found to react predominantly with the 29-kDa protein as judged by immunoblot analysis of *C. jejuni* glycine-hydrochloride extracts (results not shown). With this antiserum, an immunoreactive *E. coli* clone was identified by screening a genomic library of *C. jejuni* M275 constructed in the pBSKII$^+$ cloning vector. Whole-cell extracts of this clone were subjected to SDS-PAGE and immunoblot analysis. A 29-kDa immunoreactive band was detected in extracts of transformants containing the recombinant plasmid, designated pMEK45, and not in extracts of transformants harboring the parental pBSKII$^+$ plasmid. The pMEK45 recombinant plasmid contained an insert of 2.2 kb. A subclone of pMEK45, designated pMEK45-8, that contained a 1.1-kb insert was isolated. Subclone pMEK45-8 was also found to express the immunoreactive 29-kDa protein. Figure 1 shows a partial restriction map of pMEK45 and its derivatives.

Nucleotide and deduced amino acid sequences. Both strands of the 1,143-bp pMEK45-8 insert were sequenced and analyzed to identify potential open reading frames (ORFs). Figure 2 shows the entire sequence of the pMEK45-8 insert. Analysis of this sequence revealed the presence of a single ORF of 771 nucleotides, capable of encoding a protein of 256 amino acids with a calculated molecular mass of 28,531 Da (P29). The ORF begins with an AUG start codon at position 129 and is terminated with two in-frame stop codons, UAA and UGA, at positions 897 and 903. The proposed methionine initiation codon is preceded by a typical Shine-Dalgarno sequence (AGGA). Downstream of the two in-frame stop codons is a putative terminator sequence that has an inverted repeat preceded by 3 A's and followed by 4 U's. This sequence arrangement is characteristic of bidirectional rho-independent terminators (25, 31). The predicted isoelectric point of the P29 protein is 5.14. The G+ C content of the gene encoding the P29 protein is 30.6 mol%, which is consistent with the base composition of *Campylobacter* DNA (22).

Examination of the amino terminus of the deduced P29 amino acid sequence revealed a typical signal sequence. The N terminus of the proposed signal sequence is polar, composed of two positively charged residues (Lys), and is followed by a region of predominantly hydrophobic residues which are presumably responsible for initiating the export of the native protein across the membrane. The proposed site of cleavage of the amino-terminal leader sequence was determined by the method of Von Heijne (30) and is located between the cysteine residue at position 186 and the glutamine residue at position 189 (Fig. 2). The putative signal peptide is 20 amino acid residues long. The molecular mass of the mature protein was calculated to be 26, 328 kDa.

Determination of the transcriptional start site. The transcriptional start site of the gene encoding the 29-kDa product was determined by primer extension analysis with 30 μg of total RNA isolated from *C. jejuni* M275 and the oligonucleotide primer 5′-TTAGTGTTTTGGCAAGCCACC-3′. Extension products were electrophoresed against sequencing ladders generated with the same oligonucleotide. A single primer extension (RNase-resistant cDNA) product that had a mobility consistent with termination 27 nucleotides upstream, at an A nucleotide, of the proposed initiation codon (Fig. 3) was detected. Examination of the sequences located upstream of this site revealed putative −35 and −10 promoter sequences (TTG CCA and TATAAT, respectively) that were similar to *E. coli* −35 (TTGACA) and −10 (TATAAT) consensus sequences. The similarity in promoter sequences probably accounts for the expression of this *C. jejuni* gene in *E. coli*. An extension

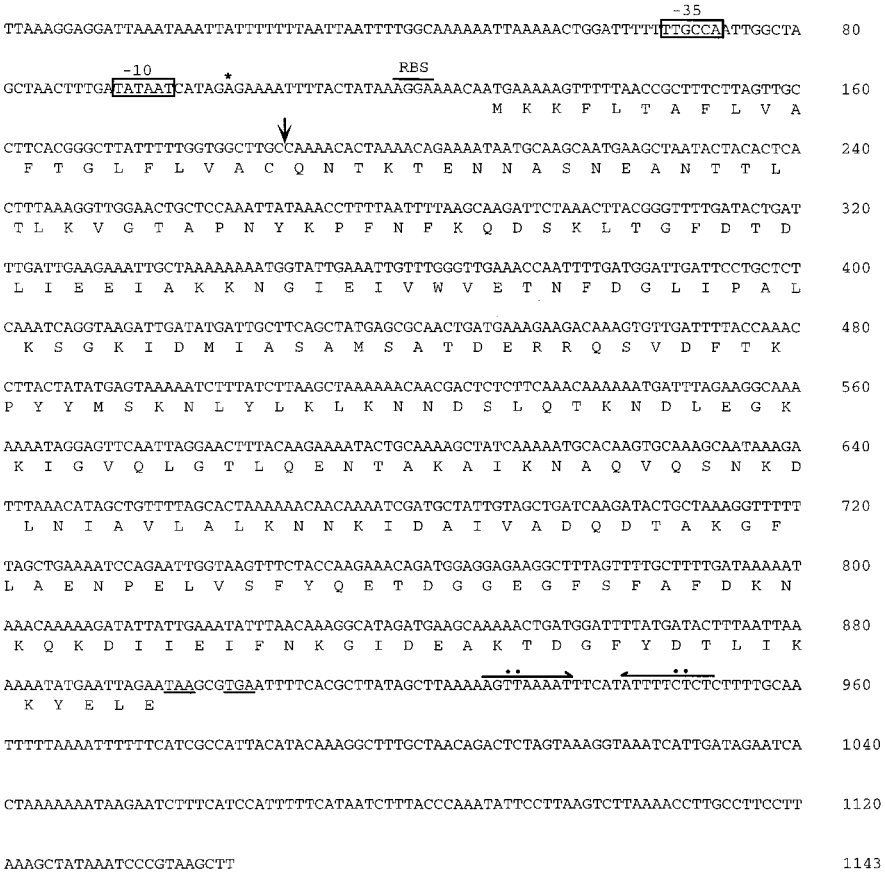

FIG. 2. Nucleotide sequence of pMEK45-8. Nucleotides are numbered from the 5′ end of the nontranscribed strand. The deduced amino acid sequence for the P29 product is given in the single-letter code below the DNA sequence. The proposed ribosomal binding site (RBS) (AGGA) is overlined. The experimentally determined transcriptional start site is indicated by an asterisk. Putative −35 (TTGCCA) and −10 (TATAAT) promoter sequences are boxed. The two in-frame translational stop codons are underlined. A possible terminator is indicated by converging arrows over an inverted repeat; the dots indicate mismatches. The proposed site of cleavage of the amino-terminal leader sequence was determined by the method of Von Heijne (30) and is located between the cysteine residue at position 186 and the glutamine residue at position 189 (vertical arrow).

product was not detected in the reactions carried out with either tRNA or no RNA (results not shown).

Expression of the 29-kDa recombinant protein in *E. coli*. Whole-cell extracts of *E. coli* transformants harboring pBSKII$^+$ or pMEK45 were subjected to SDS-PAGE and immunoblot analysis. In contrast to whole-cell extracts of transformants harboring the parental plasmid, a 29-kDa reactive band was detectable in the whole-cell extracts of transformants harboring pMEK45 as judged by immunoblot analysis with the anti-29-kDa serum (Fig. 4, lanes 1 and 2). The appearance of this band was not enhanced by the addition of isopropyl-β-D-thiogalactopyranoside (results not shown), again suggesting that the *C. jejuni* promoter is functional in *E. coli*. A 29-kDa reactive protein was also detectable by immunoblot analysis with the anti-29-kDa serum in an osmotic shock sample prepared from *E. coli* transformants harboring pMEK45 (lane 3). This finding suggests that the P29 protein is transported across the cytoplasmic membrane to the periplasmic space and that it possesses a signal sequence that is recognized in *E. coli*.

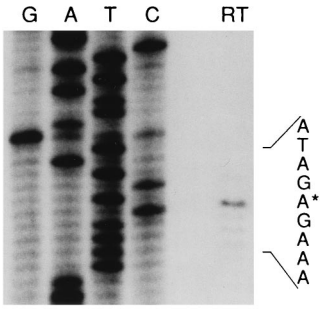

FIG. 3. Mapping of the P29 gene transcriptional start site. The transcriptional start site of the P29 gene was determined by primer extension analysis. The 21-nucleotide primer was complementary to nucleotides 51 to 71 of the P29 gene. The letters G, A, T, and C above the lanes indicate which dideoxynucleotide was used to terminate the sequencing reaction. The sequence given on the right is complementary to that which can be read from the sequencing ladder. The identity of the 5′ end of the transcript is indicated by an asterisk. The adenine residue identified was determined to be 27 nucleotides upstream of the proposed initiation codon.

To further demonstrate that the P29 was encoded by the 771-nucleotide ORF, a plasmid was constructed in which the ORF was disrupted by insertion of a 1.4-kb fragment containing a kanamycin resistance gene (*kan*). The 1.4-kb fragment was ligated into the *Cla*I site of pMEK45-8 (Fig. 1). pMEK45-8 containing the *kan* resistance gene within the ORF was designated pMEK45-8::*kan*. The 29-kDa reactive protein was detected in whole-cell extracts of *E. coli* harboring pMEK45-8 (Fig. 4, lane 4) but not in extracts of *E. coli* transformants containing pMEK45-8::*kan* (lane 5). This finding confirmed

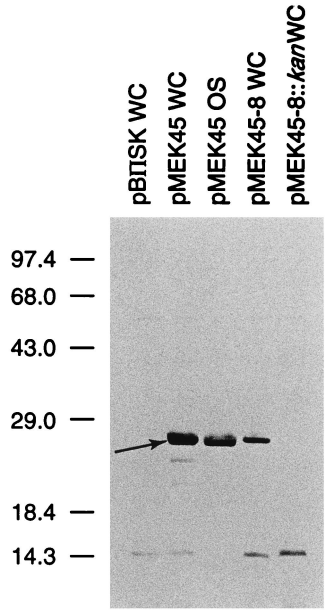

FIG. 4. Immunoblot analysis of *E. coli* XL1-Blue isolates harboring nonrecombinant and recombinant plasmids. *E. coli* extracts were resolved by SDS-PAGE (12.5% polyacrylamide), transferred to Immobilon, and reacted with the antiserum (1:250 dilution) raised against a *C. jejuni* 29-kDa gel-purified protein. Lanes: 1, whole-cell extract of *E. coli* harboring pBSKII$^+$ (pBIISK WC); 2, whole-cell extract of *E. coli* harboring pMEK45 (pMEK45 WC); 3, osmotic shock fluid of *E. coli* harboring pMEK45 (pMEK45 OS); 4, whole-cell extract of *E. coli* harboring pMEK45-8 (pMEK45-8 WC); 5, whole-cell extract of *E. coli* harboring pMEK45-8::*kan* (pMEK45-8::*kan*WC). The 29-kDa immunoreactive protein is highlighted (arrow). Molecular mass size standards, in kilodaltons, are indicated on the left.

that the 771-nucleotide ORF contained within pMEK45-8 was responsible for encoding the P29 protein.

Comparison of the P29 deduced amino acid sequence with other proteins. The P29 deduced amino acid sequence was used to search the PIR and Swiss Protein databases for similarity to other proteins. The P29 protein exhibited similarity to the HisJ (53% similar and 32% identical) and LAO (52% similar and 32% identical) periplasmic binding proteins encoded by the *hisJ* and *argT* genes of *S. typhimurium*, respectively. Figure 5 shows the alignment of the P29 deduced amino acid sequence with the *S. typhimurium* HisJ and LAO protein sequences.

Functional analysis of the P29 protein. To determine whether the P29 protein was required for the transport of arginine or histidine, complementation experiments were performed with *S. typhimurium* LAO and HisJ periplasmic binding protein mutants. The pMEK45-8 and pMEK45-8::*kan* plasmids were electroporated into the *S. typhimurium* mutants TA3286 and TA3296 (Table 1). A 29-kDa immunoreactive protein was detected in *S. typhimurium* transformants harboring pMEK45-8 but not in transformants harboring pMEK45-8::*kan*. For complementation analysis, the *S. typhimurium* mutants harboring pMEK45-8 or pMEK45-8::*kan* were inoculated into the appropriate minimal medium containing either D-histidine or L-arginine plus D-histidine. Bacterial growth was observed with the *S. typhimurium* HisJ mutant TA3296 harboring the pMEK45-8 plasmid but not with this mutant harboring the pMEK45-8::*kan* plasmid in medium supplemented with D-histidine (Fig. 6). The lag phase observed with TA3296 harboring pMEK45-8 was dependent upon the initial inoculum as determined by growth experiments in which the inoculum size was varied (results not shown). Growth was not observed with the *S. typhimurium* LAO mutant TA3286 harboring either plasmid (results not shown). The ability of the *S. typhimurium* HisJ mutant harboring pMEK45-8 to grow in minimal medium containing D-histidine, a substrate of the high-affinity histidine permease, suggests that the *C. jejuni* gene encoding the P29 protein is a homolog of the *S. typhimurium* *hisJ* gene. On the basis of these findings, the gene encoding the P29 protein has been designated *hisJ*.

Southern hybridization analysis. Southern hybridization analysis was performed to determine if a single copy of the *hisJ* gene is present in *C. jejuni* M275. *C. jejuni* M275 restricted DNA was resolved by agarose gel electrophoresis, and hybridization analysis was performed with the ^{32}P-labeled pMEK45 plasmid (results not shown). The hybridization pattern observed with each restriction endonuclease was consistent with the data obtained for the restriction profile of pMEK45. This finding indicates that this *C. jejuni* isolate contains a single copy of the *hisJ* gene.

DISCUSSION

Relatively little work has been done to characterize *C. jejuni* surface and periplasmic proteins. The reason for this may be the difficulty encountered in trying to clone and express *C. jejuni* genes in a heterologous host such as *E. coli*. In this study, we characterized one protein with an apparent molecular mass of 29 kDa that was present in glycine-hydrochloride extracts prepared from *C. jejuni*. The P29 deduced amino acid sequence was similar to the *S. typhimurium* HisJ (53% similar and 32% identical) and LAO (52% similar and 32% identical) periplasmic binding protein sequences. The deduced amino acid sequence of the *C. jejuni* P29 product also exhibited 43% similarity and 23% identity to the sequence of another *C. jejuni* protein, termed PEB1 (23). PEB1 has been proposed to mediate the attachment of *C. jejuni* to intestinal epithelial cells. The P29 product does not appear to promote the binding of *C. jejuni* to eukaryotic cells as judged by adherence assays performed with *E. coli* transformants harboring the pMEK45 recombinant plasmid; no quantitative difference was observed in the binding of transformants harboring pMEK45 and pBSKII$^+$ (results not shown). The *C. jejuni* PEB1 product has also been proposed to be involved in amino acid transport on the basis of its similarity to other nutrient transport proteins. The deduced amino acid sequence of the PEB1 product shares 28.9% identity with the *S. typhimurium* HisJ protein sequence, 22.9% identity with the *S. typhimurium* LAO protein sequence, and 27.8% identity with the *E. coli* glutamine-binding protein sequence. The genes encoding the *S. typhimurium* HisJ and LAO proteins exhibit 70% identity and are proposed to have originated from gene duplication. Given that the genes encoding the P29 and PEB1 products have only 23% identity, their functional and evolutionary relatedness to one another is unclear.

The *S. typhimurium* histidine permease is composed of a periplasmic binding protein, HisJ, and three cytoplasmic membrane-associated proteins, HisQ, HisM, and HisP (QMP complex). Similar to *S. typhimurium, C. jejuni* possesses one copy of the *hisJ* gene as judged by Southern hybridization analysis. However, the structural organization of the *hisJ* gene differs between the two organisms. In *S. typhimurium*, a single operon is responsible for encoding the periplasmic binding protein HisJ and the cytoplasmic membrane-associated proteins HisQ, HisM, and HisP. The *C. jejuni* P29 protein appears to be encoded by a monocistronic unit as determined by sequence and in vitro transcription-translation analyses. The pMEK45

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Cj P29       M K K F L T A F L V A F T G L F L V A C Q N T K T E N N A S N E A N T T L T L K V G T A P N Y K P F   50
Salty HisJ   M K K L A L S L S L V L A - - - - - - - - - - - - F S S A T A A F A A I P Q K I R I G T D P T Y A P F   39
Salty LAO    M K K T V L A L S L L I G - - - - - - - - - - - - L G A T A A S Y A A L P Q T V R I G T D T T Y A P F   39

Cj P29       N F K Q - D S K L T G F D T D L I E E I A K K N G I E I V W V E T N F D G L I P A L K S G K I D M I   99
Salty HisJ   E S K N A Q G E L V G F D I D L A K E L C K R I N T Q C T F V E N P L D A L I P S L K A K K I D A I   89
Salty LAO    S S K D A K G E F I G F D I D L G N E M C K R M Q V K C T W V A S D F D A L I P S L K A K K I D A I   89

Cj P29       A S A M S A T D E R R Q S V D F T K P Y Y M S K N L Y L K L K N N D S L Q T K N D L E G K K I G V Q   149
Salty HisJ   M S S L S I T E K R Q Q E I A F T D K L Y A A D S R L V V A K N S D I Q P T V A S L K G K R V G V L   139
Salty LAO    I S S L S I T D K R Q Q E I A F S D K L Y A A D S R L I A A K G S P V Q P T L E S L K G K H V G V L   139

Cj P29       L G T L Q E - - - N T A K A I K N A Q V Q S N K D L N I A V L A L K N N K I D A I V A D Q D T A K G   196
Salty HisJ   Q G T T Q E T F G N E H W A P K G I E I V S Y Q G Q D N I Y S D L T A G R I D A A F Q D E V A A S E   189
Salty LAO    Q G S T Q E A Y A N D N W R T K G V D V V A Y A N Q D L I Y S D L T A G R L D A A L Q D E V A A S E   189

Cj P29       F L A E N P - - - - - - - E L V S F Y Q E T D G G E G F S F A F D K N K Q K D I I E I F N K G I D E   239
Salty HisJ   G F L K Q P V G K D Y K F G G P A V K D E K L F G V G T G M G L R K E - D N E L R E A L N K A F A E   238
Salty LAO    G F L K Q P A G K E Y A F A G P S V K D K K Y F G D G T G V G L R K D - D T E L K A A F D K A L T E   238

Cj P29       A K T D G F Y D T L I K K Y - E L E - - - -                                                       256
Salty HisJ   M R A D G T Y E K L A K K Y F D F D V Y G G                                                       260
Salty LAO    L R Q D G T Y D K M A K K Y F D F N V Y G D                                                       260
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FIG. 5. Alignment of the deduced amino acid *C. jejuni* P29 sequence (Cj P29) with the *S. typhimurium* histidine (Salty HisJ) and LAO (Salty LAO) periplasmic binding proteins. Gaps, indicated by dashes, were introduced to obtain maximal alignment. Amino acids are indicated by single-letter codes and are numbered from the first methionine residue. Identical or conservative amino acids residues shared are boxed. Comparison of the *C. jejuni* P29 amino acid sequence with the *S. typhimurium* HisJ and LAO proteins yielded amino acid sequence similarities of 53 and 52%, respectively.

recombinant plasmid contains approximately 800 bp of DNA 3′ of the *C. jejuni hisJ* gene. To determine if a gene encoding one of the membrane-associated proteins was located 3′ of the *C. jejuni hisJ* gene, the proteins produced by the pMEK45 recombinant plasmid were labelled with [^{35}S]methionine by using the in vitro *E. coli* cell-free transcription-translation (S30) system (results not shown). Aliquots of the in vitro assay mixture were electrophoresed, and labelled proteins were detected by autoradiography. Analysis of the autoradiograph revealed only the insert-encoded P29 protein and vector-encoded β-lactamase. Studies are in progress to identify cytoplasmic-membrane proteins which interact with the *C. jejuni* P29 periplasmic binding protein.

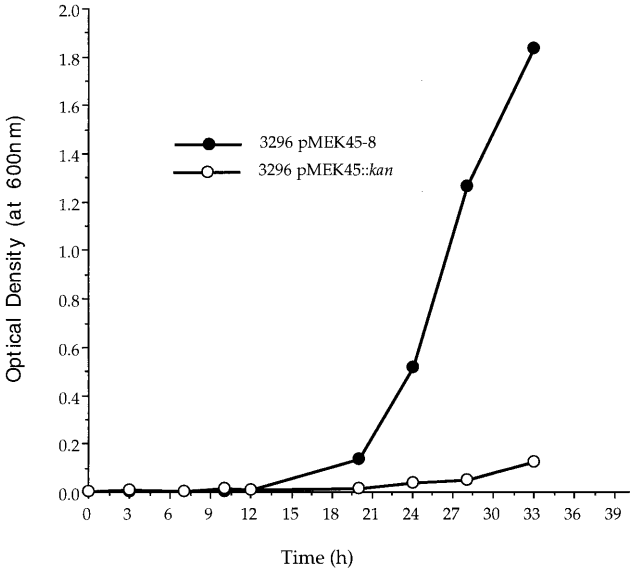

FIG. 6. Functional analysis of the *C. jejuni* gene encoding a 29-kDa protein as judged by complementation of an *S. typhimurium* HisJ mutant. *S. typhimurium* TA3296 transformants harboring pMEK45-8 or pMEK45-8::*kan* were cultured with shaking (225 rpm) at 37°C in minimal M-9 medium supplemented with 0.4% glucose, 0.001% thiamine, 10 mM ammonium chloride, 50 μg of ampicillin per ml, and 0.1 mM D-histidine. Growth was monitored turbidimetrically by measuring the optical density at 600 nm.

Few *C. jejuni* promoter sequences have been defined owing to the limited number of genes cloned. The transcription start site of the *C. jejuni hisJ* gene was determined by primer extension analysis. A single transcription start site was identified 27 nucleotides 5′ of the translational initiation codon. Inspection of the upstream sequences revealed a putative σ^{70} promoter sequence. Whether the *C. jejuni hisJ* gene is regulated by nitrogen availability, as is the case with other enteric organisms, remains to be investigated.

The *S. typhimurium* HisJ and LAO proteins possess two functionally distinct domains. One domain is involved in binding the amino acid substrate, and the other interacts with the cytoplasmic QMP membrane-bound complex. Fourteen amino acid residues which may be involved in the binding of the HisJ and LAO periplasmic binding proteins to the membrane-associated complex have been identified (10). Of these 14 amino acids, 3 were identical in the *C. jejuni* P29 and *S. typhimurium* HisJ and LAO proteins. These amino acids are at positions 85 (aspartic acid), 111 (glutamine), and 223 (lysine), using the numbering of the *C. jejuni* P29 amino acid sequence. Of the five amino acid residues that have been proposed to be involved in substrate binding (10), only one amino acid in the *C. jejuni* P29 protein was identical to the *S. typhimurium* HisJ protein. This is a threonine residue located at position 152. Collectively, four proposed key residues are identical in the *C. jejuni* P29 and *S. typhimurium* HisJ proteins. The identity of these residues suggests that they may be involved in the interaction of the periplasmic binding protein with the substrate and membrane-associated cytoplasmic proteins. Nevertheless, the lack of greater similarity or identity in residues over the

entire span of the two proteins suggests that the *C. jejuni* protein evolved under different evolutionary constraints.

In summary, we have cloned, sequenced, and characterized a *C. jejuni* gene whose product is present in glycine-hydrochloride extracts. We have designated this gene *hisJ*, on the basis of complementation experiments performed with an *S. typhimurium* HisJ mutant harboring the pMEK45-8 recombinant plasmid which contains a 2.2-kb fragment of *C. jejuni* chromosomal DNA. These data represent the first step in the identification and characterization of *C. jejuni* periplasmic binding proteins involved in amino acid transport. Additional studies are required to identify the cytoplasmic membrane components involved in histidine uptake in *C. jejuni*.

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