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Identification, Purification, and Characterization   Army Project Order
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Identification, Purification, and Characterization of Major Antigenic Proteins of *Campylobacter jejuni**

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Evidence from developing countries and volunteer studies indicates that immunity to *Campylobacter jejuni* and *Campylobacter coli* may be acquired, but the antigenic basis for this protection is poorly defined. We have purified to homogeneity four proteins with molecular weights of 28,000 (PEB1), 29,000 (PEB2), 30,000 (PEB3), and 31,000 (PEB4) from epidemic *C. jejuni* strain 81–176 using acid extraction and sequential ion-exchange, hydrophobic interaction, and gel filtration chromatography. The relative amino acid compositions of these four proteins are similar. NH_2-terminal sequence analysis indicates that all four proteins are different, although the first 35 amino acids of PEB2 and PEB3 are 51.4% homologous. Isoelectric focusing showed that all four are basic proteins with pI of 8.5 for PEB1 protein and >9.3 for the others. Use of the purified proteins as antigens in an IgG enzyme-linked immunosorbent assay (ELISA) found that seroconversion to the PEB1 or PEB3 proteins occurred in 15 of 19 patients with sporadic *C. jejuni* or *C. coli* infection. In comparison, only two, six, and 14 of these patients seroconverted to PEB2, PEB4, or the acid extract antigen. In an ELISA with whole bacterial cells as antigens, antiserum to the acid-extracted antigens showed broad recognition of *C. jejuni, C. coli, C. fetus, C. lari,* and *Helicobacter pylori*. Antiserum to PEB1 recognized all 35 *C. jejuni* and all 15 *C. coli* strains but none of the isolates of the other three bacterial species. The PEB1 and PEB3 proteins appear to be candidate antigens for both a *Campylobacter* vaccine and for serological assays for the pathogen.

Campylobacter jejuni and the closely related species *Campylobacter coli* are now recognized as important causes of acute diarrheal disease in humans throughout the world (1–3). *C. jejuni* and *C. coli* are frequently present in the intestinal tracts of the animals we use for food processing, and transmission to humans from consumption of undercooked poultry and raw milk is common (4–7). Control of these pathogens in both human and animal populations would be desirable.

Several lines of evidence suggest that protective immunity can be developed against these pathogens. The attack rate for diarrheal disease with *C. jejuni* is decreased in adults in developing countries (8, 9) and in chronic drinkers of raw milk in the United States (10) suggesting that immunity is induced by recurrent reexposure. Studies in human volunteers have shown that experimental infection with *C. jejuni* induces serum and intestinal antibodies directed against the pathogen, and also protects against subsequent illness (but not infection) upon rechallenge with the same strain (11).

However, while exposure to these bacteria may induce a protective host response (8–11), the pathogenicity of live *C. jejuni* and *C. coli* limits the utility of a whole cell vaccine. A vaccine containing *C. jejuni* antigens that were presented to the recipients in a non-virulent manner would be preferable. This could be accomplished using a vaccine composed solely of purified *C. jejuni* antigens. However, there is considerable heterogeneity of both heat-stable and heat-labile antigens (12). Nevertheless, serum antibody response to *C. jejuni/coli* in infected persons has been assessed using crude materials extracted with glycine at low pH (8, 13), but the precise antigenic basis of this reactivity is poorly understood.

For both development of vaccines and serological testing, characteristics of the common antigen present in this mixture would be useful. The flagella of *C. jejuni* are present in this extract and have been found to contain major antigens, but these proteins are heterogeneous and subject to phase variation (14, 15). Several studies have suggested that a *C. jejuni* protein migrating at approximately $M_r = 30,000$ by SDS-PAGE[1] is common, cell surface-exposed, and antigenic to humans (16–19). Because several *C. jejuni* proteins migrate at approximately this molecular weight, purification of the proteins is necessary to determine if a single common antigen truly exists. We have now purified four *C. jejuni* proteins between $M_r = 28,000$ and 31,000 and have shown that while they are similar in amino acid compositions, they differ in amino-terminal sequence. Two of the four proteins are commonly recognized by convalescent sera from patients with sporadic *C. jejuni/coli* enteritis. These two proteins may be candidates for a vaccine against *Campylobacter* enteritis and also may be of value in serological assays for the diagnosis of this infection.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—A *C. jejuni* strain 81–176 originally isolated from an outbreak of *C. jejuni* diarrhea and shown to be virulent in human volunteers and non-human primates

[1] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

(19, 20), was used as the source for antigen preparation. This strain was maintained frozen at −70 °C in brucella broth (BBL Microbiology Systems, Cockeysville, MD) containing 15% glycerol and has been deposited in the American Type Culture Collection (ATCC 55026). For purification of *C. jejuni* proteins, the bacterial strain was grown on trypticase soy agar with 5% sheep blood (PASCO, Wheat Ridge, CO) in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, and 85% nitrogen) at 37 °C for 24 h for three generations. Other strains including 38 *C. jejuni*, 20 *C. coli*, five *Campylobacter fetus*, five *Campylobacter lari*, and five *Helicobacter pylori* strains studied were from either the *Campylobacter* strain library of Vanderbilt University or from the Centers for Disease Control (CDC) *Campylobacter* laboratory provided by Charlotte Patton. Of the CDC strains, nine of 11 *C. jejuni*, all nine *C. coli*, and all five *C. lari* strains have been identified to the species level by DNA hybridization. These strains had also been maintained at −70 °C in brucella broth containing 15% glycerol and for studies were also grown on trypticase soy agar with 5% sheep blood in a microaerobic atmosphere at 37 °C.

Purification of C. jejuni Proteins with HPLC— For isolation of the antigens, bacterial cells were harvested and washed with cold distilled water by centrifugation at 8000 × g for 10 min. A crude mixture of surface proteins was extracted with 0.2 M glycine hydrochloride buffer, pH 2.2, as previously described (21). The preparation was lyophilized and reconstituted with distilled water and desalted using a Sephadex G-15 (Pharmacia LKB Biotechnology Inc.) column with distilled water as running buffer. For final purification of proteins, the crude mixture was separated by hydrophobic interaction chromatography performed on a phenyl-Superose column (Pharmacia), ion-exchange chromatography on a Mono S column (Pharmacia), or gel filtration chromatography on a Superose 12 column (Pharmacia) using a fast protein liquid chromatography system (Pharmacia) (Fig. 1). The column eluates were monitored for UV absorbance at 280 nm to define protein peaks. Fractions were checked for presence and purity of specific antigens using SDS-PAGE analysis performed with 0.75-mm thick gels in a mini-Protean II dual slab cell (Bio-Rad) at 250 mA for about 40 min.

Analytic Procedures— Protein concentrations were measured using a BCA protein assay kit (Pierce Chemical Co.) for crude proteins, and using Quantigold (Diversified Biotech, Newton Center, MA) for the purified proteins. SDS-PAGE was performed in a modified Laemmli gel system as described by Ames (22). Proteins were resolved using the modified silver stain of Oakley *et al.* (23). Molecular mass standards (Bio-Rad) and their weight in daltons were: phosphorylase b (97,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,000). The isoelectric point of the proteins was determined by isoelectric focusing in Resolve™ thin layer agarose gels with a pH range of 3 to 10 (IsoLab, Inc., Akron, OH). Protein samples (0.5 μg) were focused under native conditions and resolved using the silver stain of Willoughby and Lambert (24). An experient formula of pI *versus* migration distance was generated by using four standard proteins of known pI between 8.3 and 9.3 as follows: L-lactic dehydrogenase (8.3, 8.4, and 8.6) and trypsinogen (9.3) (Sigma). Analysis for the presence of disulfide bonds in the purified proteins was performed by treating the proteins with 0.9% dithiothreitol at 37 °C for 30 min in SDS sample buffer (1.57% Tris base, 4.0% SDS, 20% glycerol, and 0.0025% bromphenol blue, pH 6.8), and monitoring molecular weight change by SDS-PAGE in comparison to untreated proteins.

Western blotting was performed by the method of Towbin *et al.* (25). After SDS-PAGE separation, proteins were transferred to nitrocellulose paper by electroblotting, the nitrocellulose paper was incubated for 30 min in Tris/saline blotting buffer (TSBB) (10 mM Tris base, pH 8.0, 0.5 M NaCl, 0.5% Tween 20, 0.02% NaN₃), then incubated for 60 min with primary rabbit antiserum at a 1:2000 dilution in TSBB. After three washes in TSBB, the nitrocellulose paper was incubated for 60 min with 1:2000 dilution of alkaline phosphatase-conjugated anti-rabbit IgG (Amersham Corp.). After washing, the nitrocellulose paper was developed in substrate solution containing 0 ml of 3 mM MgCl₂ in 50 mM Tris, pH 10.0, 1 ml of 0.1% nitroblue-tetrazolium and 0.1 ml of 0.5% of 5-bromo-4-chloro-3-indoxylphosphate (Sigma) in dimethyl formamide.

Production of Antiserum— Antisera to the crude acid extract mixture and the M_r 28,000 antigen (PEB1) of strain 81-176 were raised in adult New Zealand White female rabbits by three subcutaneous injections of 5 μg of protein as previously described (26).

Detection of Antibody Response in Human Patients— The purified antigens were compared with the previously described crude acid-

extracted preparation for utility as antigens for sero-diagnosis of *Campylobacter* infection (13). A standardized enzyme-linked immunosorbent assay (ELISA) with 20 ng of protein ELISA well was used to detect human anti-*Campylobacter* antibodies. Sera obtained from patients who sought attention at medical facilities in Denver, Colorado (27) for acute diarrheal illness were studied. Acute phase sera had been obtained within 7 days of illness onset and convalescent sera had been obtained 11–40 days later. Seroconversion was defined as an optical density value in convalescent serum that was at least 50% greater than that in the paired acute phase serum.

Digestion of PEB1 Antigen with Proteases— For exoprotease digestion with proteinase K (Boehringer Mannheim) 24-h cultures of *Campylobacter* strains on blood agar plates were harvested in sterile distilled water (5 ml/plate). The cells were pelleted at 3500 × g for 10 min, resuspended in water, and protein concentration determined using the BCA protein assay kit and adjusted to 240 μg/ml with water. 100 μl of bacterial suspension was incubated for 60 min at 37 °C with Proteinase K (2.4 μg) (or water alone as control). 100 μl of SDS-sample buffer added, the samples boiled for 5 min, and then electrophoresed on SDS-PAGE with 15% acrylamide. For digestion with endoprotease protease V8 (Boehringer Mannheim), the crude acid extract mixtures were dialyzed against water to remove glycine, denatured by boiling in 2% SDS for 5 min, and then diluted 1:10 with 0.1 M NaHCO₃. Either Protease V8 or water was added to these denatured samples at ratios up to 1:4 of enzyme/bacterial proteins (w/w), and the samples incubated for up to 48 h at 37 °C. The samples then underwent SDS-PAGE and Western blotting with rabbit antiserum to PEB1.

Determination of Native Molecular Weight of PEB1 Antigen— The native molecular weight of PEB1 antigen was determined in a Superose 12 (Pharmacia) gel filtration column using gel-filtration molecular weight markers (Sigma) as follows: horse spleen apoferritin (443,000), sweet potato β-amylase (200,000), yeast alcohol dehydrogenase (150,000), bovine erythrocyte carbonic anhydrase (29,000), and horse heart cytochrome c (12,400). Blue dextran (2,000,000) was used to determine the void volume. Individual protein standards were dissolved in an equilibration buffer containing 50 mM Tris-HCl, 100 mM KCl, pH 7.5. Crude acid-extract mixtures of *C. jejuni/coli* proteins were dialyzed against water to remove glycine and concentrated using Centricon-10 microconcentrators (Amicon, Danvers, MA). The first water extracts of *C. jejuni/coli* strains were concentrated in the same way. Each of these samples were diluted 1:1 with the Tris-KCl buffer, then either 50 μl of sample or a molecular weight protein standard was loaded onto the column. Elution volume of the standards was individually determined by the position of the absorption peak at 280 nm. A standard curve for molecular weight determination was generated by a semilog regression of the elution volumes *versus* the log₁₀ molecular weights of the individual protein standards. The elution volume of PEB1 antigen was determined by assaying for the presence of the M_r = 28,000 band in each fraction using SDS-PAGE and Western blotting with rabbit anti-PEB1.

Identification and Differentiation of Isolates of Campylobacter and Helicobacter by ELISA— 35 *C. jejuni*, 15 *C. coli*, 10 *C. fetus*, five *C. lari*, and five *H. pylori* strains were used as antigens in this study. The bacteria were grown overnight, harvested in distilled water, and protein concentrations adjusted to 1 μg/μl. Whole bacterial cells (0.5 μg protein/well) were then used in an IgG ELISA (13). The specific antisera were absorbed with whole *Escherichia coli* cells to remove any antibodies to *E. coli* that may cross-react with *Campylobacter* and diluted (1:500 for antisera to PEB1 protein and for the acid-extracted preparation) prior to use in the assay. An optical density value greater than 0.1 was defined as positive.

Identification of Campylobacter by Western blot with Antiserum to PEB1— Whole bacterial cells were prepared as described above, and SDS-PAGE performed on a 15% acrylamide gel using 0.5 μg of bacterial protein/lane, and then Western blot analysis performed as described above.

Amino Acid Analysis and Amino-terminal Sequencing— Purified proteins were prepared by dialysis against water and lyophilization. Amino acid analysis was performed using the method of Jones (28). Amino-terminal sequencing was performed on an Applied Biosystems 470 A Protein Sequencer equipped with a 120A autoanalyzer using the 03RPTH program, as previously described (26).

RESULTS

Purification of C. jejuni Antigens—Whole bacterial cells and crude acid-extracted mixtures from four *C. jejuni* strains

isolated from 1978 to 1987 were compared (data not shown). Major proteins in the acid-extracted preparations migrated at about 62, 30, 28, 24, and 21 kDa. The protein profiles of both whole cells and acid-extracted mixtures were very similar, although the strains had been isolated over a 10-year period. Because of its well-characterized virulence despite multiple *in vitro* passages (11), we started our purification with the crude acid-extract mixture from strain 81–176. The purification of the PEB antigens is summarized in Fig. 1. PEB1 was purified to greater than 98% homogeneity by a single passage of the acid extract preparation on a phenyl-Superose column that fractionated material into three major protein peaks (Fig. 2). Peak 1 represented hydrophilic materials that did not bind to the column at pH 9.0. Peak 2 contained an essentially pure mixture of $M_r = 30,000$ (PEB3) and 31,000 (PEB4) antigens. Peak 3 contained purified PEB1 antigen that migrated at 28,000 in SDS-PAGE. The precise elution point of peaks 2 and 3 varied with the age of the column, eluting at approximately $0.3–0.5$ M Na_2SO_4 in a new column (Fig. 2) and $0.0–0.1$ Na_2SO_4 in a much used column (data not shown). However, the SDS-PAGE profile of each peak remained similar and the third peak always contained pure PEB1. The PEB2 ($M_r = 29,000$), PEB3 ($M_r = 30,000$), and PEB4 ($M_r = 31,000$) antigens were partially purified by cation-exchange separation on a Mono S column (Fig. 3). PEB3 eluted at approximately 180 mM NaCl (*peak 3*), PEB4 at approximately 200–220 mM NaCl (*peak 3* + *peak 4*), and PEB2 at approximately 300 mM NaCl (*peak 7*). PEB3 was further purified to homogeneity on a phenyl-Superose column as originally used for PEB1. The PEB4 was purified to homogeneity by passing the partially purified material through a Superose 12 gel filtration column with 0.15 M phosphate, 0.15 M NaCl, pH 7.2, at a flow rate of 0.5 ml/min. For PEB2, the partially purified antigen from the Mono S column was further purified to homogeneity on the phenyl-Superose column as for PEB1, followed by a Superose 12 column, as for PEB4 antigen. The final purified products are shown in Fig. 4.

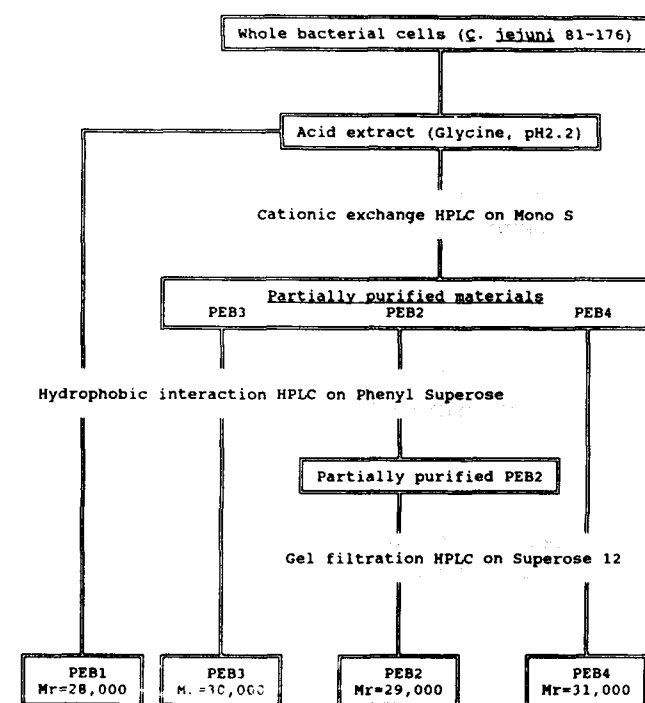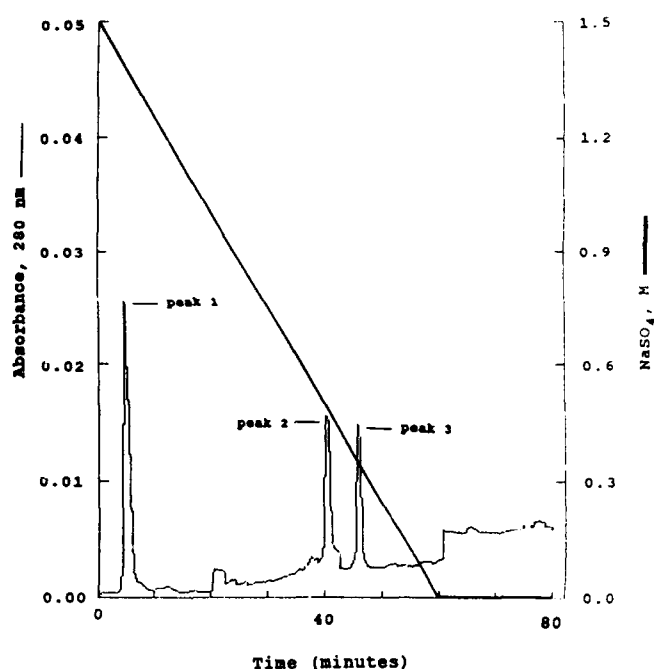*Seroconversion to C. jejuni Proteins in Patients with Acute*

FIG. 2. **Purification of PEB1 antigen from *C. jejuni* strain 81–176 by hydrophobic interaction fast protein liquid chromatography on a phenyl-Superose column.** Elution was with 20 mM borate, pH 9.0, with a linear decrease of Na_2SO_4 concentration from 1.5 to 0.0 M over 60 min at a flow rate of 0.25 ml/min. Fractions of eluted materials were collected every 2 min and assayed for the presence of PEB1 by SDS-PAGE. *Peak 3* contained purified PEB1.

Diarrheal Illness by ELISA—The purified proteins were then compared with the crude acid-extracted mixture from strain 81–176 in an ELISA to detect IgG responses in patients with sporadic cases of diarrhea (Table I). Of 19 patients with *C. jejuni* or *C. coli* diarrhea, 14 seroconverted to the acid-extracted mixture, 15 to PEB1 or PEB3, two to PEB2, and six to PEB4. None of patients without *Campylobacter* diarrhea seroconverted to any of these antigens.

Digestion of the PEB1 Antigen with Proteases—Since bacterial antigens may be polysaccharide, lipopolysaccharides, or protein in nature, we tested whether PEBs are proteins to further characterize these antigens. Although previous experiments showed that PEB1 and PEB3 were common antigens, we were unsuccessful in purifying sufficient PEB3 due to its instability at temperatures above 0 °C. Thus, the following characterizations only focused on PEB1 antigen. Whole cells of two *C. jejuni* and two *C. coli* strains and an acid extract from a third *C. jejuni* strain were digested with proteinase K or V8. Rabbit antiserum to PEB1 was used in Western blot to monitor the sensitivity of PEB1 to the protease digestions. This antiserum is monospecific and recognized a single antigen band migrating at $M_r = 28,000$ in whole cell preparations, indicating that no degradation of the protein occurred during the extraction and purification processes. The $M_r = 28,000$ band disappeared after proteinase K digestion but not after sham digestion (data not shown), indicating that the major antigenic component of PEB1 is a protein. PEB1 was fully resistant to digestion with protease V8 at low enzyme concentration (2% enzyme for 24 h at 37 °C). Continuation of digestion with 25% enzyme for another 24 h partially hydrolyzed PEB1 into two antigenic fragments that migrate at $M_r = 16,000$ and 12,000 (data not shown).

Determination of the Native Molecular Weight of the PEB1 Antigen—Two *C. jejuni* (strains 81–176 and D191b) and two *C. coli* (strains D743 and D1035) strains were used in this

FIG. 1. **Schematic purification of PEB proteins from *C. jejuni* strain 81–176.**

FIG. 3. **Purification of PEB2, PEB3, and PEB4 proteins from acid extract of strain 81–176 by cation-exchange chromatography on a Mono S column with 50 mM HEPES, pH 9.0, at a flow rate of 1 ml/min and a linear gradient from 0 mM NaCl to 400 mM NaCl over a period of 20 min.** Peaks contained partially purified proteins, PEB3 (*peak 3*), PEB4 (*peak 3 + 4*), and PEB2 (*peak 7*).
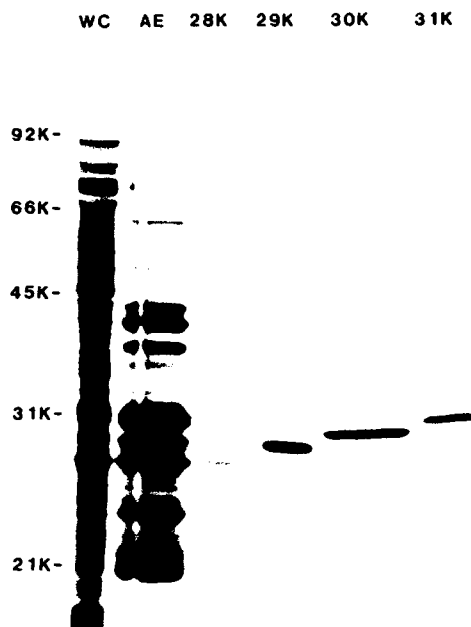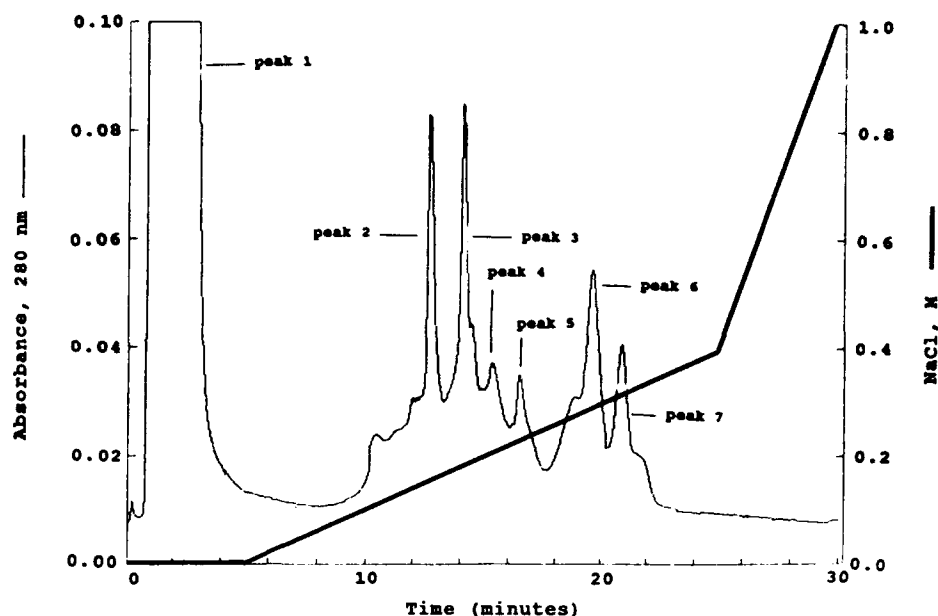

FIG. 4. **SDS-PAGE (with 12% acrylamide) of purified *C. jejuni* proteins from strain 81–176.** The lanes are whole bacterial cells (*WC*), acid extract (*AE*), PEB1 (*28K*), PEB2 (*29K*), PEB3 (*30K*), and PEB4 (*31K*). Molecular weight markers are shown at left.

study. Crude acid-extract mixtures were chromatographed on the Superose 12 column. PEB1 consistently eluted from the column immediately after the molecular weight standard protein carbonic anhydrase ($M_r = 29,000$) (data not shown), and had a calculated molecular weight of 28,900. To compare the effect of extraction conditions on polymerization of PEB1, we included water extract preparations in this study. Only trace amounts of PEB1 was extracted in water, so the water extract was concentrated 50-fold before use. PEB1 extracted in water was also found to have a molecular weight of $28,900 \pm 1,000$ for each of four strains tested. The similarity in the calculated molecular weight of PEB1 as defined by SDS-PAGE and gel filtration chromatography indicates that the native form of PEB1 is a monomer of $M_r = 28,900 \pm 1,000$. PEB1 in *C. jejuni* and *C. coli* strains have the same native molecular weights.

Identification of Campylobacter and Helicobacter Strains by Antiserum to C. jejuni by ELISA—We next examined the potential application of antibodies to *C. jejuni* proteins for identification and diagnosis of *Campylobacter* and/or *Helicobacter* species. Rabbit antisera to the acid-extract mixture of strain 81–176 and to PEB1 were used in this study. By IgG ELISA we tested for the presence of cross-reactive antigens in 35 *C. jejuni* strains, 15 *C. coli*, 10 *C. fetus*, five *C. lari*, and five *H. pylori* strains. The control normal rabbit serum did not recognize any of the isolates tested. Antiserum to the acid-extract mixture recognized all 35 *C. jejuni*, all 15 *C. coli*, nine of 10 *C. fetus*, all five *C. lari*, and three of five *H. pylori* strains. In contrast, the antiserum to PEB1 recognized all 35 *C. jejuni* and all 15 *C. coli* isolates but none of the isolates of the other three bacterial species (Fig. 5). Thus, antiserum to PEB1 appeared to have exceptional discriminatory power having both 100% sensitivity and specificity for *C. jejuni* and *C. coli*.

Detection of PEB1 in Whole Bacterial Cells by Western Blot—To further confirm the specificity of recognition of antiserum to PEB1, we performed Western blotting to determine the bands recognized in preparations of whole cells of various *Campylobacter* and *Helicobacter* species. In total, 18 *C. jejuni* strains, 14 *C. coli*, three *C. fetus*, four *C. lari* strains, and one *H. pylori* strain were tested. A $M_r = 28,000$ band was found in all 18 *C. jejuni* and all 14 *C. coli* strains but was not present in any of the *C. fetus*, *C. lari*, or *H. pylori* strains tested (Fig. 6). *C. jejuni* strains consistently had higher OD values in ELISA than *C. coli* (Fig. 5) and denser bands at $M_r = 28,000$ in the Western blot analysis (Fig. 6). This Western blot experiment provided physical evidence that PEB1 from various *C. jejuni/coli* strains are all conserved in size, antigenically related, and can be recognized by antiserum to PEB1 antigen from a single strain, 81–176 (ATCC55026). However, the results also suggest that either more PEB1 is present in *C. jejuni*, or that there are slight antigenic differences between PEB1 of *C. jejuni* and *C. coli*. The amino-terminal residues of PEB1 from *C. jejuni* and *C. coli* are 70% identical (Fig. 7); the amino acid differences are consistent with antigenic differences.

Determination of pI—Prior to isoelectric focusing, the purified antigens were dialyzed against water and concentrated

TABLE I

Seroconversion to C. jejuni proteins as determined by ELISA of serum from Campylobacter infected persons and persons with other diarrheal diseases

Seroconversion is defined as OD_{414} value increase by at least 50% in convalescent serum compared with that in acute serum and optical density value in convalescent serum greater than 0.200 in ELISA.

Patient	Isolate	AE	PEB1	PEB2	PEB3	PEB4
	C. jejuni/coli					
1.	*C. coli*	+	+	−	+	−
2.	*C. coli*	+	+	−	+	+
3.	*C. jejuni*	−[a]	+	−	−[a]	+
4.	*C. coli*	−[a]	..[a]	−[a]	−[a]	−[a]
5.	*C. jejuni*	−	−	−	+	−
6.	*C. jejuni*	+	+	−	+	+
7.	*C. jejuni*	+	+	−	+	−
8.	*C. coli*	+	+	−	+	−
9.	*C. jejuni*	+	+	−	+	−
10.	*C. jejuni*	+	+	−[a]	+	−
11.	*C. coli*	+	+	−	+	−
12.	*C. jejuni*	+	+	+	+	+
13.	*C. jejuni*	+	+	−	+	−
14.	*C. jejuni*	+	+	−	+	+
15.	*C. jejuni*	−	−	−	+	−
16.	*C. jejuni*	+	+	−	+	−
17.	*C. jejuni*	+	+	+	+	+
18.	*C. jejuni*	+	+	−	−	−
19.	*C. jejuni*	−[a]	−	−	−[a]	−
% seroconversion		73.7	78.9	10.5	78.9	31.6
	Other pathogens					
1.	*Shigella*	−	−	−	−	−
2.	*Salmonella*	−	−	−	−	−
3.	*Shigella*	−	−	−	−	−
4.	*Yersinia*	−	−	−	−	−
5.	*Salmonella*	−	−	−	−	−
% seroconversion		0	0	0	0	0
	No pathogens identified					
1.		−	−	−	−	−
2.		−	−	−	−	−
3.		−	−	−	−	−
4.		−	−	−	−	−
5.		−	..	−	−	−
% seroconversion		0	0	0	0	0

[a] Optical density value greater than 1.0 in both acute and convalescent serum. Patients with acute serum OD value greater than 1.0 were not considered to show seroconversion, regardless of OD values in convalescent serum.
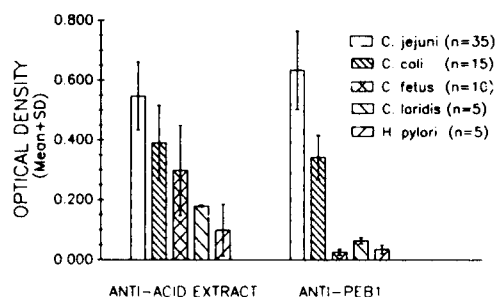

FIG. 5. **Recognition of *Campylobacter* and *Helicobacter* cells by antisera to *C. jejuni* proteins by ELISA.** Whole bacterial cells were used as antigens. First antibodies were rabbit anti-acid extract or rabbit anti-PEB1 from strain 81-176. An optical density value at 414 nm greater than 0.1 was defined as positive (mean ± S.E. of two experiments).

using centricon-10 microconcentrators (Amicon). All four proteins were found to be basic with a pI of 8.5 for PEB1, and pI greater than 9.3 for PEB2, PEB3, and PEB4.

Amino Acid Composition and Amino-terminal Sequence—

a b c d e f g h i j k l m n o

28→

FIG. 6. **Western blot of rabbit anti-PEB1 with representative *Campylobacter* and *Helicobacter* strains.** The antigens used are whole cells prepared as described in the text. Bacterial strains of *C. jejuni* (except strains 81-176, 81-93, and 81-94), *C. coli*, and *C. lari* had been identified by DNA hybridization to the species level. The *C. fetus* strains were identified by the presence of high molecular weight surface array proteins detected by SDS-PAGE and Western blot (25). The method for the Western blot is as described in the text. The *arrow* indicates bands migrating at $M_r = 28,000$, which were found in all *C. jejuni* strains (81-176, 81-93, 81-95, D996, and D1916, *lanes a–e*) and all *C. coli* strains (D743, D1035, D130, D126, and D115, *lanes f–j*) but not found in any of *C. lari* strains (D459 and D1014, *lanes k* and *l*), *C. fetus* strains (84-32 and 80-109, *lanes m* and *n*) or *H. pylori* strain (16-11A, *lane o*).

The amino acid composition of the four proteins was found to be similar (Table II). Not surprisingly, basic amino acids (Lys, His, and Arg) predominate, ranging from 26.4 to 33.6%. Cysteine was not found in any of the four proteins. Treatment with dithiothreitol did not change the electrophoretic migration of the four proteins (data not shown), confirming the absence of intermolecular disulfide bonds. A comparison of the NH₂-terminal amino acid sequences of the four proteins indicated that each is unique, although there is 51.4% identity in the NH₂-terminal 35 amino acids of PEB2 ($M_r = 29,000$) and PEB3 ($M_r = 30,000$) (Fig. 7). None of the NH₂ termini of these mature proteins starts with methionine, suggesting that a leader sequence was cleaved during maturation. The four amino acid sequences were compared with several GeneBank databases including PIR version 25.0, Swiss-Prot 14, EMBL 24, and unique GeneBank sequences 65-24. The NH₂ termini of PEB1 or PEB2 had no significant in-frame homology with other known proteins. For PEB3, we identified a potential in-frame homology with the deduced protein sequences of two bacterial structures related to adherence to eukaryotic cells, class I pili from *Neisseria meningitidis* (30) and *E. coli* heat-labile enterotoxin B-subunit (31). PEB4 was found to share an identical NH₂-terminal sequences through the first 30 amino acids with an $M_r = 31,000$ protein purified from a *C. jejuni* strain VC74 (29) (Fig. 7). PEB4 also exhibits partial homology with two proteins related to the serine protease inhibitor superfamily including human corticosteroid-binding globulin (32), serine proteinase inhibitor I of vaccinia virus (33, 34), and with two mammalian proteins involved in cell-to-cell interaction including mouse neural cell adhesion molecule (35) and human MHC class II lymphocyte antigen Dpw4-β-1 (36).

DISCUSSION

We have developed methods for the purification of four basic proteins from *C. jejuni* strain 81-176 and demonstrated the existence of common antigens, migrating between $M_r = 28,000$ and $31,000$ by SDS-PAGE. Although no more than four protein bands of whole cells and acid-extract of *C. jejuni* are distinguishable between $M_r = 28,000$ and $31,000$ by one-dimensional SDS-PAGE, more than 10 protein spots are resolved around $M_r = 30,000$ by two-dimensional gel electrophoresis with pI between 4.0 and 6.8 (37). The four basic PEB

FIG. 7. **Amino-terminal sequence of *C. jejuni* and *C. coli* proteins.** Identical residues are *highlighted in bold lettering*. Dash indicates that residue was not determined. [a]M_r = 28,000 protein from *C. coli* strain D1035. [b]Sequence of M_r = 31,000 proteins of VC74 in Ref. 29.

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         1        5        10        15        20        25        30        35        40
PEB1    G E G K L E S I K S K G Q L I V G V K M D V P H Y A L - D Q A - - -
D1035[a] A E G K L E A I K A K G E L V I G V K N
PEB2    E I L V Y G P G G P A P V L K E L A L K F E E K T K E K V I V T A G P
PEB3    D V N L Y G P G G P H T A L K D I A S K Y S E K T G V K V N V N F G P Q A T F - K
PEB4    A T V A T V N G K S I S D A E V S E F F A P M L R G Q D F K - L - D
VC74[b] A T V A T V N G K S I S D A E V S E F F A P M L R G Q D F K T L P - N Q K A
```

TABLE II

Amino acid composition of C. jejuni proteins

Amino acid residue[a]	PEB1	PEB2	PEB3	PEB4	VC74 protein M_r = 31,000[b]
	mol %				
Lys	30.2	24.4	22.9	32.4	14.3
His	0.6	0.0	1.4	0.3	1.1
Arg	2.0	2.0	4.0	0.9	1.1
Asx	11.1	14.1	11.9	11.8	13.6
Glx	6.6	9.1	7.5	11.4	15.8
Ser	3.8	4.9	4.6	2.9	4.0
Thr	4.4	3.9	6.7	5.6	5.0
Val	5.5	5.5	5.4	4.1	5.0
Met	0.4	1.7	1.5	0.8	1.1
Ile	5.2	5.8	6.0	4.1	3.4
Leu	7.4	7.7	5.1	6.4	7.4
Ala	9.4	11.1	9.3	7.4	9.7
Phe	3.3	2.5	4.6	3.7	4.7
Gly	6.7	5.1	6.3	5.7	7.2
Cys	0.0	0.0	0.0	0.0	0.0
Tyr	3.5	2.1	2.8	2.4	0.0

[a] Trp and Pro not determined.
[b] From Ref. 29.

proteins found in the present studies raise the number of proteins around this region to at least 14. It is important to recognize that the presence of several antigenic proteins with similar molecular weights clustering around M_r = 30,000 make it impossible to identify the major antigen based only on molecular weight. The four purified proteins share the following characteristics. 1) All are removable from the bacterial cell by a gentle extraction with low pH glycine, suggesting they are not transmembrane proteins. 2) They are similar in amino acid compositions and contain a high percentage of lysine. The difference in lysine value (Table II) between PEBs and VC74 protein suggests that the unusual high percentage of lysine in PEBs may be caused by contamination. 3) All are basic proteins with pI greater than 8.5. 4) Amino acid analysis and unchanged migration in SDS-PAGE after treatment with a reducing agent indicates that all lack cysteine. 5) None of the amino termini of the mature proteins start with methionine, indicating post-translational cleavage of a leader sequence. However, in spite of these similarities, they differ in amino-terminal sequences and in their ability to stimulate the immune response during natural infections. The close homology between PEB2 and PEB3 may be due to gene duplication during evolution, but its significance is not known.

The strongly cationic nature of these proteins is striking and may aid in anchoring the proteins within the membrane through electrostatic interaction with negatively charged lipopolysaccharide molecules or other outer membrane components. The cation charge also may aid in interactions with eukaryotic cells. Of interest, the *mip* protein of *Legionella pneumophila*, an M_r = 24,000 surface protein which enhances the ability of *L. pneumophila* to parasitize human macrophages and to cause pneumonia in experimental animals, has a similarly high pI of 9.8 (38, 39).

Dubreuil and co-workers (29) have purified a M_r = 31,000 protein from *C. jejuni* which has sequence identity for the first 30 amino acid with PEB4. Although this protein is

common to thermophilic *Campylobacter*, and antigenic when used in pure form to immunize rabbits, our data indicate that it is not a major target protein for the human immune system during natural infection. Digestion of *Campylobacter* cells with trypsin did not remove this protein (29), suggesting that PEB4 is not surface-exposed. Whether failure to stimulate an antibody response to this protein during natural infection is due to its internal location or to other characteristics is unclear. Work to define the location of the other three proteins on *Campylobacter* cells is presently underway in our laboratory and will hopefully provide more data to correlate location and antigenicity during natural infections.

In the present study, two proteins, PEB1 and PEB3, have been found to be common antigens recognized by convalescent sera from either *C. jejuni*- or *C. coli*-infected patients. PEB1 is found in all *C. jejuni* and *C. coli* strains. PEB3 share significant sequence homology with two other bacterial proteins, class I pilin from *N. meningitidis* and heat-labile enterotoxin B-subunit of *E. coli*, both of which are involved in binding to human mucosal cells, although at present the significance of the homologies is unknown. Work by other investigators has shown that *C. jejuni* proteins of about M_r = 30,000 participate in bacteria-host cell interactions (40). Adherence of these molecules to tissue culture cells were only found for invasive strains. A M_r = 28,000 protein may play a role in *C. jejuni* colonization in chicken (41). However, these molecules have only been identified by molecular weight. Without more specific identification, we cannot directly compare these molecules with the PEBs, but the similarity in molecular weight between these molecules and PEBs and sequence homology between PEB3 and other adhesive molecules suggests a relationship between them.

C. jejuni, C. coli, and C. lari are enteric bacterial that colonize the small intestine where a family of serine proteases such as trypsin and chymotrypsin are normally present in high concentrations. Resistance of *Campylobacter* cells to this digestion may be important for survival but no mechanism has been established. Digestion of whole *C. jejuni* cells with trypsin did not remove an M_r = 31,000 protein (PEB4) (29), and we found that soluble acid-extracts from *C. jejuni* and *C. coli* strains were highly resistant to digestion with another serine protease, proteinase V8. PEB4 shares significant in-frame homology with members of the serine protease inhibitor superfamily (32–34). Thus, it may be worthwhile to assay PEB4 directly for protease inhibitor activity. Finding such activity could explain the conservation of PEB4 among all intestinal *Campylobacters*.

Campylobacter are microaerophilic and fastidious to culture. Currently available methods for detection of *C. jejuni* and *C. coli* require special media and incubation conditions and are time consuming. Rapid direct detection of infectious agents using immunological methods is becoming widely used (42), but little has been done for *C. jejuni* and *C. coli*. Two kinds of assays could be useful in diagnosis: (i) use of antibodies to PEBs to identify *C. jejuni* or *C. coli* in clinical specimens and cultures; and (ii) use of PEBs as antigens to detect antibody response during natural infections. Antibodies to PEB1 are potentially suitable for rapid identification of bacterial cells,

and PEB1 and PEB3 appear to be good candidate proteins to detect antibody responses.

Clinical and epidemiologic studies suggest it may be possible to develop an effective vaccine for *C. jejuni* and *C. coli* (8–11). Since diseases caused by *C. jejuni* and *C. coli* are self-limited, and recovery is usually associated with development of antibodies to specific antigens on the bacterial cell surface (13), one approach to vaccine development is to identify and characterize these cell surface molecules. We have found two proteins, PEB1 and PEB3 that are targets for the human immune system during natural infection. Whether or not the immune response to PEB1 and PEB3 is protective is unknown at present. Further study needs to focus on localization of the PEBs on the bacterial cell surface, and their role in bacterial adherence and pathogenesis, and the efficiency of PEB1 or PEB3 for active immunization or their antibodies for passive protection of animals experimentally infected with *C. jejuni* or *C. coli*.

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