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Identification and Characterization of an Immunogenic Outer Membrane Protein of *Campylobacter jejuni*

ANDRÉ BURNENS, URS STUCKI, JACQUES NICOLET, AND JOACHIM FREY*

Institute for Veterinary Bacteriology, University of Berne, CH-3012 Berne, Switzerland

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We cloned and expressed in *Escherichia coli* a gene encoding an 18-kDa outer membrane protein (Omp18) from *Campylobacter jejuni* ATCC 29428. The nucleotide sequence of the gene encoding Omp18 was determined, and an open reading frame of 165 amino acids was revealed. The amino acid sequence had the typical features of a leader sequence and a signal peptidase II cleavage site at the N-terminal part of Omp18. Moreover, the sequence had a high degree of similarity to the peptidoglycan-associated outer membrane lipoprotein P6 of *Haemophilus influenzae* and the peptidoglycan-associated lipoprotein PAL of *E. coli*. Southern blot analysis in which the cloned gene was used as a probe revealed genes similar to that encoding Omp18 in all species of the thermophilic group of *campylobacters* as well as *Campylobacter sputorum*. All campylobacters tested expressed a protein with a molecular mass identical to that of Omp18. The protein reacted immunologically with polyclonal antibodies directed against Omp18 from *C. jejuni*. PCR amplification of the gene encoding Omp18 with specific primers and subsequent restriction enzyme analysis of the amplified DNA fragments showed that the gene for Omp18 is highly conserved in *C. jejuni* strains isolated from humans, dogs, cats, calves, and chickens but is different in other *Campylobacter* species. In order to obtain pure recombinant Omp18 protein for serological assays, the cloned gene for Omp18 was genetically modified by replacing the signal sequence with a DNA segment encoding six adjacent histidine residues. Expression of this construct in *E. coli* allowed purification of the modified protein (Omp18-6×His) by metal chelation chromatography. Sera from patients with past *C. jejuni* infection reacted positively with Omp18-6×His, while sera from healthy blood donors showed no reaction with this antigen. Omp18, which is an outer membrane protein belonging to the family of PALs is well conserved in *C. jejuni* and is highly immunogenic. It is therefore a good candidate as an antigen for the serological diagnosis of past *C. jejuni* infections.

The thermophilic *Campylobacter* species, particularly *Campylobacter jejuni* and *Campylobacter coli*, are recognized as important causes of acute diarrheal disease in humans throughout the world (37). Studies in human volunteers have shown that experimental infection with *C. jejuni* induces serum and intestinal antibodies directed against the pathogen (4). Extraintestinal complications of *C. jejuni* infection such as reactive arthritis, pancreatitis, and carditis were described (29). A recent work provides evidence that a few *C. jejuni* strains are capable of triggering an immunologic response with antigenic characteristics that stimulates the production of antibodies which in turn react with peripheral nerve myelin, causing the Guillain-Barré syndrome (18). For both the development of vaccines and serological analysis, conserved surface antigens of *C. jejuni* will be of great importance.

Only a few surface antigens of *Campylobacter* species, however, have been characterized genetically and immunologically so far. These include the flagellin and protein PEB1 of *C. jejuni*. Their genes have been cloned and sequenced (12, 16, 26). The antigenicity and the immunodominance of flagellins during infection are well established (23, 25, 27). However, there is accumulating evidence that the immunodominant epitopes are serotype restricted (25). Furthermore, the flagellar filament can undergo antigenic variation (1) and the *flaA* genes show considerable genetic diversity among different *Campylobacter* strains (2, 21). The biochemical and antigenic properties of the *Campylobacter* flagellar hook protein revealed a protein which is surface exposed and immunogenic in humans (9, 30). Recently, two antigenic proteins, PEB1 and PEB3, which are commonly recognized by convalescent-phase sera from patients with sporadic *C. jejuni* diarrhea were identified. PEB1 was determined to be a major cell adherence factor which is conserved in all *C. jejuni* and *C. coli* isolates. PEB3 was not characterized further (10, 26, 27). The major outer membrane proteins of *C. jejuni* and *C. coli*, which are probably porins, are surface exposed and seem to be immunogenic to some extent during infection (25).

Here we report the cloning and analysis of an immunogenic 18-kDa outer membrane protein, Omp18, of reference strain *C. jejuni* ATCC 29428 which exhibits considerable similarity to peptidoglycan-associated lipoproteins (PALs) of *Haemophilus influenzae* and *Escherichia coli*. This surface protein seems to be a common, *Campylobacter*-specific antigen and is a good candidate for the development of assays for the serological diagnosis of past *C. jejuni* infections.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The bacterial strains used in the study are listed in Table 1. *Campylobacter*, *Bacteroides*, *Arcobacter*, and *Helicobacter* spp. were cultured on Columbia agar plates supplemented with 5% sheep blood (Bio Mérieux, Geneva, Switzerland) at 37°C in a microaerophilic atmosphere consisting of 6% O_2, 7% CO_2, and 7% H_2 in N_2 for 20 h. For all gene cloning and expression experiments *E. coli* Sure (Stratagene, La Jolla, Calif.) or *E. coli* BL21(DE3) (36) (a gift from Stan Tabor, Harvard Medical School, Boston, Mass.) was used. *H. influenzae* type b strain B 51 was obtained from T. Omland, Oslo, Norway (11).

Plasmids pBluescriptIISK$^-$ and pBluescriptIIKS$^+$ (Stratagene), pET-15b (Novagen, Madison, Wis.), and pQE-16, (Qiagen, Chatsworth, Calif.) were used to clone the genomic DNA fragments of *C. jejuni*. *E. coli* strains were grown in Luria-Bertani broth (LB) (32). Media were supplemented with 25 μg of ampi-

* Corresponding author. Mailing address: Institute for Veterinary Bacteriology, University of Berne, Länggassstrasse 122, CH-3012 Berne, Switzerland. Phone: 41 31 631 24 84. Fax: 41 31 631 26 34. Electronic mail address: JFREY@VBI.UNIBE.CH.

TABLE 1. Bacterial strains used in the study

Bacteria	Strain no.[a]	Reference	Source
Campylobacter jejuni	ATCC 29428	Reference strain	Human infant
Campylobacter jejuni	NCTC 11351	Type strain	Bovine
Campylobacter jejuni	NCTC 11828	Reference strain	Human
Campylobacter jejuni Lior 131	D 759-90	Clinical isolate	Dog
Campylobacter jejuni Lior 71	E 1830-91	Clinical isolate	Feline
Campylobacter coli	LMG 6440	Type strain	Porcine
Campylobacter coli Lior 12	CCUG 12073	Serotype reference strain	Human
Campylobacter lari	NCTC 11352	Type strain	Avian (herring gull)
Campylobacter lari Lior 35	CCUG 15035	Reference strain	Sea gull
Campylobacter upsaliensis	LMG 8850	Reference strain	
Campylobacter helveticus	NCTC 12470	Type strain	Feline
Campylobacter fetus subsp. *fetus*	ATCC 25936	Type strain	Ovine
Campylobacter hyointestinalis	ATCC 35217	Type strain	Porcine
Campylobacter mucosalis	ATCC 43264	Type strain	Porcine
Campylobacter sputorum biovar *sputorum*	ATCC 35980	Type strain	Human
Campylobacter rectus	ATCC 33238	Type strain	Human
Bacteroides ureolyticus	ATCC 33387	Type strain	Human
Arcobacter butzleri	ATCC 49616	Type strain	Human
Helicobacter pylori	ATCC 43504	Type strain	Human
Helicobacter fennelliae	NCTC 11612	Type strain	Human
Arcobacter pleuropneumoniae	ATCC 27089	Serotype 2	Swine
Haemophilus influenzae	B 51	Type b	Human
Escherichia coli XL1 blue			

[a] ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, United Kingdom; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; LMG, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium.

cillin per ml for the selection of pBluescriptIISK$^-$ or 25 μg of tetracycline per ml for the propagation of *E. coli* Sure.

Extraction of DNA, gene cloning, and colony screening procedures. DNA from *Campylobacter* strains was extracted by a slightly modified standard procedure (28). The addition of 0.1 volume of a 3 M NaCl solution instead of ammonium chloride was used to promote the precipitation of DNA. Nucleic acid concentrations were estimated on agarose gels, with bacteriophage lambda DNA used as a reference. Competent *E. coli* cells were prepared by the calcium chloride method (32). For screening, colonies were transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany). The filters were then incubated for 4 h at 37°C on LB agar plates supplemented with 25 μg of ampicillin per ml and 166 μM isopropyl-β-D-thiogalactopyranoside. The filters were soaked in lysis buffer (100 mM Tris-HCl [pH 7.8], 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin, 50 μg of lysozyme per ml) for 4 h and were incubated with hyperimmune serum raised against *C. jejuni* and developed as described previously (3).

DNA sequencing and analysis. The sequence analysis was accomplished by the dideoxy chain termination method (33) with [^{35}S]dATP (Amersham International plc, Amersham, United Kingdom) and T7 DNA polymerase with the Sequenase sequencing kit (United States Biochemicals, Cleveland, Ohio). The two standard oligonucleotide primers T3 and T7 (Stratagene) were used, and the following two oligonucleotides were synthesized (Microsynth, Windisch, Switzerland) for use in sequencing: P320 (5′-GTTGAATTTATCAAAATC-3′) at positions 456 to 439 and Ag4-772 (5′-CGATGAGTGGGGAACCG-3′) at positions 549 to 565 of the DNA sequence in Fig. 1B. The DNA sequences were assembled and analyzed by using the PCGENE DNA and protein analysis programs (University of Geneva, Geneva, Switzerland).

Southern blotting and DNA hybridization. Genomic DNAs from different bacterial strains were digested with *Hin*dIII. DNA fragments were separated on 0.7% agarose gels, depurinated in 0.25 M HCl for 10 min, denatured in 0.5 M NaOH–1 M NaCl for 60 min, neutralized in 1 M Tris-HCl (pH 7.5)–3 M NaCl for 30 min, transferred to nitrocellulose Hybond-C Extra membranes (pore size, 0.45 μm; Amersham plc) by capillary transfer (32), and fixed by exposing the membranes for 2 min to UV light (32). The DNA probes were labelled with [^{32}P]dCTP (3,000 Ci/mmol; Amersham plc) by using a random primed DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Hybridizations were carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7.0])–50% formamide–5% polyethylene glycol 6000–0.5% sodium dodecyl sulfate (SDS)–100 μg of denatured and sonicated salmon sperm DNA per ml for 24 h. The membranes were washed in 1× SSC–0.1% SDS at room temperature for 30 min. Autoradiography was carried out for 20 h on Fuji RX films with an intensifying screen.

PCR. PCR was performed with a thermal cycler (9600; Perkin-Elmer Cetus, Norwalk, Conn.). Two microliters of genomic bacterial DNA at a concentration of approximately 1 μg/ml was added to 100 μl of the PCR mixture (10 mM Tris-HCl [pH 8.3], 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.005% Tween 20, 0.005% detergent Nonidet P-40, 170 μM [each] deoxynucleoside triphosphate including 0.25 μM [each] oligonucleotide primer). A total of 2.5 U of *Thermus aquaticus* DNA polymerase (Boehringer Mannheim) was added to each reaction mixture. The samples were subjected to 35 cycles of amplification consisting of denaturation at 94°C for 1 min, primer annealing at 46°C for 1 min, and chain extension at 74°C for 1 min. The oligonucleotide primers (Microsynth) used were CJPAL1-L (5′-ccgctcgagGTTATTAGTGGTT GTAGCA-3′) corresponding to nucleotides 295 to 313 and CJPAL2-R (5′-ccgctcgagTTATCTTGATAATTTAAATTC-3′) corresponding to nucleotides 750 to 730. The nucleotides of the primers represented in lowercase letters were added to create restriction enzyme sites for *Xho*I.

Protein electrophoresis and immunological analysis. Whole bacterial cells were suspended in 200 μl TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) to a concentration of 10^9 cells per ml and were solubilized by boiling for 5 min in an equal volume of SDS probing buffer (62.2 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue). The outer membrane proteins of *C. jejuni* were obtained by a rapid miniprocedure (5). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (13). Electrophoresis was performed with a stacking gel of 5% acrylamide, a separation gel of 12.5% acrylamide, and a constant current of 160 mA. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, Calif.) by electrophoretic blotting with transfer buffer (20% methanol, 186 mM glycine, 25 mM Tris, 0.01% SDS) in a Midget Multibox 2051 Electrophoretic Transfer Unit (Pharmacia LKB Biotechnology, Uppsala, Sweden). After transfer, the nitrocellulose membranes were treated with washing buffer (10 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 0.5% Tween 20, 0.01% merthiolate, 1% dried milk powder). The membranes were reacted for 90 min with serum diluted 1:2,000 in washing buffer. Bound antibodies were demonstrated by using a phosphatase-conjugated antibody against rabbit immunoglobulin G (Kirkegaard Perry Inc., Gaithersburg, Md.) in a substrate dilution buffer (10 mM sodium carbonate-bicarbonate [pH 9.6], 1 mM $MgCl_2$) including 1/100 volume of the substrates BCIP (5-bromo-4-chloro-3-indolyl phosphate; 15 mg/ml in dimethylformamide) and NBT (Nitro Blue Tetrazolium; 30 mg/ml in 70% dimethylformamide).

Production of antigens and antisera. To produce a hyperimmune serum against whole cells of *C. jejuni* ATCC 29428, a standard protocol (15) was followed.

To produce monospecific anti-Omp18 rabbit antibodies, we produced a genetically modified, polyhistidine-tailed protein named Omp18-6×His. For this purpose, the gene encoding Omp18 was amplified by PCR with the primers CJPAL1-L and CJPAL2-R. The amplified DNA fragment was then cloned into the *Xho*I site of the cloning vector pET-15b (Novagen), thus constructing a gene expressing a fusion protein named Omp18-6×His with the amino acids MetGly SerSerHisHisHisHisHisHisSerSerGlySeuValProArgGlySerHis instead of the leader sequence at its N terminus. This fusion protein contains six consecutive histidine residues for Ni^{2+}-chelate affinity chromatography; this is followed by a thrombin cleavage site for further processing. The constructed gene was overexpressed by induction of the T7 RNA polymerase in *E. coli* BL21(DE3) ac-

cording to the supplier's protocol. The cells were lysed with 6 M guanidine hydrochloride, and the histidine-tagged protein Omp18-6×His was purified by Ni^{2+}-chelate affinity chromatography according to standard protocols of the suppliers (Qiagen Inc.). Pooled fractions were further purified by SDS-PAGE by isolating the stained protein band by electroelution (Biotrap; Schleicher & Schuell). Rabbits were immunized subcutaneously with 150 μg of the purified protein Omp18-6×His mixed 1:1 with complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). An equal quantity of Omp18-6×His was injected after 4 weeks. The serum was collected 10 days after administration of the booster injection.

Sera from nine patients suffering from arthritis after an episode of campylobacter-associated gastroenteritis reacting positively in a commercial complement binding reaction with *C. jejuni* antigens (Virion, Rüschlikon, Switzerland) were a gift from A. Keller (Virion). Sera from 50 healthy blood donors without signs of gastroenteritis in the preceding 2 weeks were obtained from the blood donation service (Swiss Red Cross, SRK, Berne, Switzerland).

Nucleotide sequence accession number. The GenBank-EMBL accession number of the sequence is X83374.

RESULTS

Cloning and analysis of a *C. jejuni* outer membrane protein gene. A gene bank of the reference strain *C. jejuni* ATCC 29428 was made by cloning partially *Sau*3A-digested DNA into the *Bam*HI-*Bgl*II sites of cloning vector pQE-16 in *E. coli* Sure. Colonies were screened with hyperimmune serum directed against live *C. jejuni* ATCC 29428 bacteria by the protocol described by Ausubel et al. (3). Several clones expressing *C. jejuni* proteins with sizes ranging between 15 and 100 kDa were identified. A clone containing a protein with an apparent molecular mass of 18.5 kDa, named Omp18, which strongly reacted on immunoblots with the same serum sample, was retained. Mouse antiserum which was made by immunization with total cells of the *E. coli* clone expressing Omp18 reacted with a protein with an apparent molecular mass of 18 kDa on immunoblots with *C. jejuni*. The slightly lower molecular mass found in *C. jejuni* compared with the protein expressed in *E. coli* could be due to posttranslational cleaving and processing in campylobacters. In addition, the recombinant Omp18 protein showed a reaction with sera from patients convalescing from a *C. jejuni* infection. The recombinant plasmid of this clone, pIVB6A, was therefore analyzed further. The insert DNA from pIVB6A was extracted as a 1.8-kbp *Eco*RI-*Bgl*II fragment and was cloned into the *Eco*RI-*Bam*HI sites of pBluescriptIIKS$^+$ to give plasmid pIVB6 (Fig. 1A). Individual fragments of the 1.8-kbp *Eco*RI-*Bgl*II fragment were subcloned onto pBluescriptIIKS$^+$ or pBluescriptIISK$^-$ as outlined in Fig. 1A. Two subclones, subclones pIVB11 and pIVB12, expressed Omp18, while the others did not, showing that Omp18 is encoded on the 1-kbp *Hin*dII-*Xba*I segment (Fig. 1A). In order to analyze whether expression of Omp18 in these plasmids was promoted by the vector, the Ω-Spc fragment, which contains a strong transcription stop signal (7), was inserted between the p_{lac} promoter of the vector and the cloned insert. No difference in the expression of Omp18 was observed in *E. coli* strains harboring pIVB11 or pIVB12 or the pIVB11:Ω-Spc or pIVB12:Ω-Spc derivatives, respectively, indicating that the gene encoding Omp18 was expressed in *E. coli* from an endogenous *C. jejuni* promoter sequence on the cloned fragment.

The DNA sequences of the *Hin*dII-*Xba*I fragments of pIVB6 from the different subclones shown in Fig. 1 were determined by using the universal T3 and T7 primers and by using internal primers P320 and Ag4-772 to ensure double-strand sequencing. The DNA sequence is shown in Fig. 1B. The nucleotide sequence has an open reading frame potentially encoding a protein of 165 amino acids with a calculated molecular mass of 17.8 kDa. It is preceded by a consensus sequence for a ribosome binding site (34) and by several potential promoter sequences. Two additional incomplete open reading frames are on the same strand, and no open reading frames longer than 35 amino acids are on the opposite strand. Analysis of the DNA sequence-deduced amino acid sequence of Omp18 by using the Prosite Programme from PCGENE revealed a leader sequence and a signal peptidase II cleavage site with the cysteine residue at position 19 (Fig. 1B). Comparison of the amino acid sequence of Omp18 with sequences in the Swiss Prot Data Bank by using the BLAST programs of the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md., revealed 36% identical amino acids with the peptidoglycan-associated outer membrane lipoprotein P6 of *H. influenzae* (24) and 32% identical amino acids with the peptidoglycan-associated membrane protein PAL of *E. coli* (14). The amino acid sequence of Omp18 shows a serine residue at position 20 following the cysteine residue at the signal sequence peptidase II cleavage site (Fig. 1B). This sorting signal (40) indicates that Omp18 is an outer membrane protein of *C. jejuni.*

Genetic and serological similarities of Omp18 in *C. jejuni* isolates and in various *Campylobacter* and related species. Southern blot analysis of *Hin*dIII-digested DNA from all strains listed in Table 1 were hybridized with a probe of the Omp18 gene which was prepared by PCR amplification with primers CJPAL1-L and CJPAL2-R and subsequently labelled with [α-^{32}P]dCTP. A strongly hybridizing band of 1.5 kbp was found in *C. jejuni* (Fig. 2). Weaker hybridization signals with bands of different sizes were observed for *C. coli*, *C. lari*, *C. upsaliensis*, *C. helveticus*, and *C. sputorum*, while other *Campylobacter* species and other bacteria used as controls did not react with the probe.

In order to estimate the genetic conservation of Omp18 in *C. jejuni* isolates, the gene encoding Omp18 (*omp18*) was amplified by using the same primers, primers CJPAL1-L and CJPAL2-R. Forty-one fresh clinical isolates of *C. jejuni* from different sources, humans, dogs, cats, calves, and chickens, were used for the gene amplification experiment. All strains revealed a 450-bp fragment upon amplification. After digestion of the amplified DNA with *Dra*I, *Hin*dIII, and *Hin*fI they all showed the same fragments with sizes as expected from the DNA sequence, illustrating that the *omp18* gene is conserved within this species. The primers CJPAL1-L and CJPAL2-R also amplified a DNA fragment of a similar size in *C. coli* and *C. helveticus* strains. Digestion of the amplified DNA fragments with the same restriction enzymes listed above showed distinct and conserved patterns for each species.

The immunologic reactions of whole-cell extracts of all strains listed in Table 1 on immunoblots with antiserum against recombinant Omp18-6×His are shown in Figure 3. Strains of all *Campylobacter* species reacted with a protein with an apparent molecular mass of 18 kDa. Of the related organisms, only *Bacteroides ureolyticus*, which may belong to the genus *Campylobacter* (39), *Arcobacter butzleri*, and *Helicobacter fennelliae* reacted with proteins of different sizes in the same range. The immunologic reactions were much more conserved than the hybridization signals. This is not surprising in view of the overall conservation of the functionally important PALs among gram-negative bacteria.

Localization of Omp18 in the outer membrane and immunogenicity in humans. The amino acid sequence of Omp18, as deduced from the DNA sequence of *omp18*, indicates that this protein is an outer membrane protein. To confirm this, outer membrane protein fractions of *C. jejuni* ATCC 29428 were reacted in immunoblots with anti-Omp18-6×His antibodies. Anti-Omp18-6×His antibodies strongly reacted with the 18-kDa protein in the outer membrane protein fraction, while they did not react with fractions of inner membrane proteins.

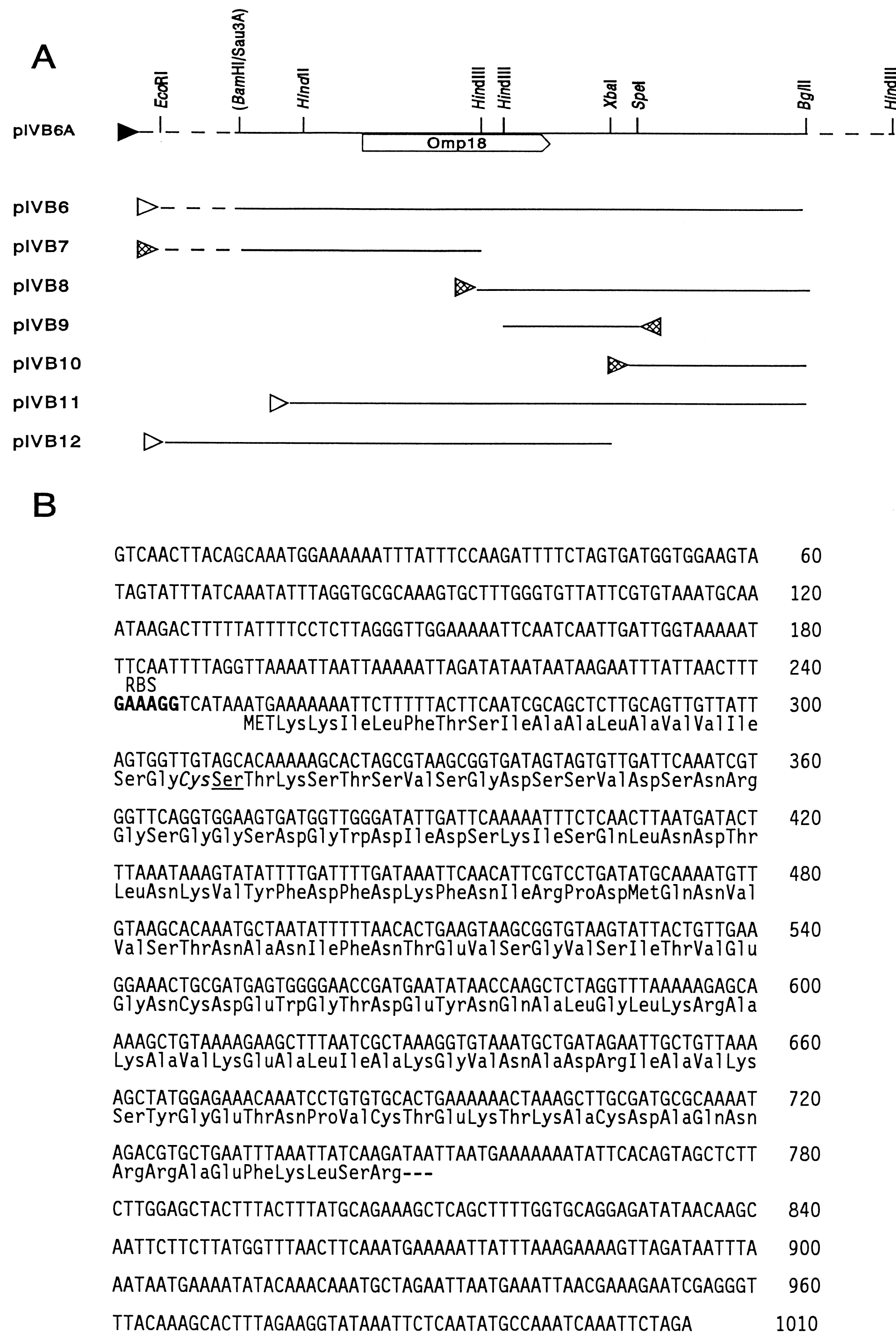

FIG. 1. (A) Restriction enzyme maps of plasmid pIVB6A and derivatives thereof and localization of the *omp18* gene. Full lines represent insert DNA from *C. jejuni* ATCC 29428; broken lines represent parts of the vectors used. The arrowheads indicate the position of the vector's p_{lac} promoters: filled arrowheads, in pQE16; hatched arrowheads, in pBluescript SK$^-$; open arrowheads, in pBluescript KS$^+$. (B) Nucleotide sequence of the *Hin*dIII-*Xba*I segment of plasmid pIVB6A. The consensus sequence for the ribosome binding site (RBS) is indicated. The signal sequence's peptidase cleavage site at the cysteine residue is indicated in italics, and the amino acid representing the sorting signal is underlined. The DNA sequence accession number is X83374.

Antibodies also recognized Omp18 in the outer membrane protein fraction which had been previously heated to 100°C for 15 min, but they did not react with an aliquot of the outer membrane protein fraction which had been incubated with 100 ng of proteinase K for 60 min at 37°C prior to loading on the SDS-polyacrylamide gel.

Sera from humans which had a past *C. jejuni* infection were analyzed. Of the nine patient serum samples reacting with *C. jejuni* in a commercially available complement binding reaction, seven gave a distinct reaction with purified recombinant Omp18-6×His protein on immunoblots, as shown in Fig. 4. In contrast, only 1 of 50 serum samples from healthy blood do-

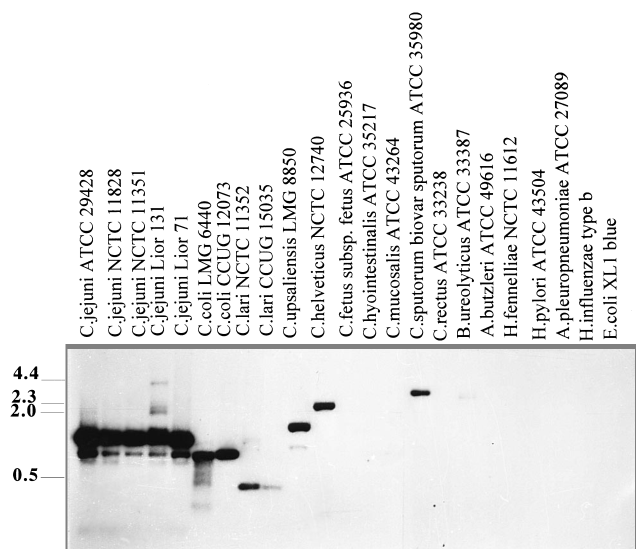

FIG. 2. Southern blot with genomic DNAs of all type strains of the thermophilic *Campylobacter* group as well as phylogenetically closely related and more distantly related strains with a labelled probe of the *omp18* gene. Bacteriophage lambda DNA digested with *Hin*dIII was used as a marker; the sizes of the standards are given on the left (in kilobase pairs).

nors showed a weak reaction with the Omp18 on immunoblots (Fig. 4, lane 10).

DISCUSSION

Infections with *C. jejuni* are known to be able to cause extraintestinal complications such as reactive arthritis and pancreatitis (29). Clinical and epidemiological evidence has suggested that infection with *C. jejuni* may be a precipitating factor for the development of the Guillain-Barré syndrome (18). The development of a reliable serodiagnostic marker for the sero-

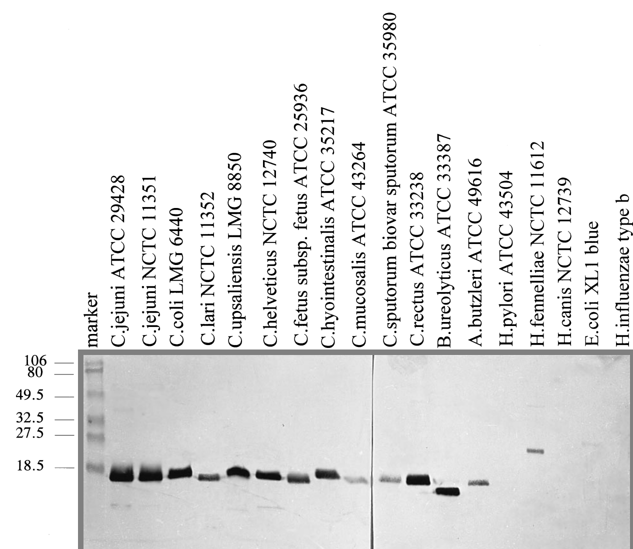

FIG. 3. Immunoblot analysis of various *Campylobacter* species and a few other closely and distantly related bacterial species, which were used as controls, with rabbit antiserum against recombinant Omp18-6×His. The molecular mass standards (prestained standards; Bio-Rad) (marker lane) are indicated on the left (in kilodaltons).

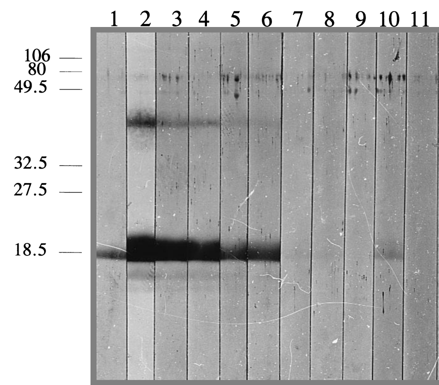

FIG. 4. Serological reactions of sera from humans with a past *C. jejuni* infection to Omp18-6×His. Lane 1, hyperimmune serum raised against whole cells of the reference strain *C. jejuni* ATCC 29428 diluted 1:1,000; lane 2, anti-Omp18-6×His rabbit serum diluted 1:2,000; lanes 3 to 6, convalescent-phase patient sera diluted 1:50; lanes 7 to 11, a selection of five representative serum samples (of 50 serum samples tested) from healthy blood donors diluted 1:50. The molecular mass standards (prestained standards; Bio-Rad) (marker lane) are indicated on the left (in kilodaltons).

diagnosis of past *C. jejuni* infections is important for epidemiological studies and for revealing the role of *C. jejuni* in causing extraintestinal complications. We have cloned and expressed in *E. coli* the gene encoding an immunogenic outer membrane protein of 18 kDa, Omp18, of the reference strain *C. jejuni* ATCC 29428 and have shown that it is a suitable candidate antigen for serological investigations. The DNA sequence of the cloned DNA fragment containing the *omp18* gene was determined, and the deduced amino acid sequence revealed a significant homology, with 36% of the amino acid sequence being identical to the amino acid sequence of the PAL (outer membrane protein P6) of *H. influenzae* (24) and 32% of the amino acid sequence being identical to the amino acid sequence of the PAL of *E. coli* (14) over the entire length of the protein. Related proteins have been described in a wide variety of bacterial species (6, 8, 14, 17, 24, 35, 38), in which they generally play an important role in the host immune response. In addition, PAL P6 of *H. influenzae* is suggested to be an important antigen for the induction of protective immunity (7, 20). The amino acid sequence of Omp18 shows a consensus sequence for a precursor signal sequence and a cysteine residue at position 19 which is known to be the cleavage site for signal sequence peptidase II (31). Moreover, the amino acid following the cysteine residue at the cleavage site is a serine residue. It had been shown that the amino acid at this position serves as a sorting signal for the final localization of the protein and that the serine residue is generally a signal for the protein to be finally translocated to the outer membrane (19, 31, 40). The localization of Omp18 in the outer membrane of *C. jejuni* was confirmed with anti-Omp18 antibodies which reacted specifically with preparations of outer membrane proteins and not with proteins from an inner membrane faction. Immunofluorescence of *C. jejuni* with fluorescence-labelled anti-Omp18 antibodies confirmed that the protein is expressed on the surface (data not shown). It must be noted that the mature Omp18 protein in *C. jejuni* has an apparent molecular mass of 18 kDa, which is higher than the molecular mass calculated from the amino acid sequence of the peptide after signal sequence peptidase cleavage. This might be due to further post-translational modifications of Omp18 or of an alternative signal cleavage site in *Campylobacter* species, which does not seem very probable to us in view of the high degrees of amino

acid identity in the signal sequence. Although we have not demonstrated directly that Omp18 is peptidoglycan associated, the high degree of amino acid homology with PAL of *E. coli* and P6 of *H. influenzae* and its location in the outer membrane suggest that Omp18 belongs to the family of PALs. This is further supported by a particularly high degree of amino acid identity between Omp18 and PAL at the C-terminal end, which is suggested to be necessary for the binding of PAL to the peptidoglycan layer (14).

Southern blot analysis revealed that genes which show similarity to *omp18* are detected in the thermophilic group of *Campylobacter* species, including *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*, and related genes with less similarity were detected in *C. sputorum* and *A. butzleri*. PCR amplification and subsequent restriction fragment analysis of the coding sequence for Omp18 from a large number of *C. jejuni* isolates from various hosts revealed that the *omp18* gene is conserved in this species. Immunoblot analysis with monospecific anti-Omp18 antibodies confirmed that similar proteins are expressed in all *Campylobacter* species.

In order to obtain highly pure recombinant Omp18 for immunological and serological experiments, the cloned *omp18* gene was genetically manipulated in order to get a modified Omp18-6×His protein devoid of the signal sequence for peptidase II but containing six histidine residues at its N-terminal end fused to residue 15 (valine). This protein was overexpressed in *E. coli* and was purified from the soluble cytoplasmic fraction by metal affinity chromatography. In our experiments it showed the same serological properties as native Omp18. This protein induced good monospecific polyclonal anti-Omp18 antibodies in rabbits and was also used for serological analysis for the detection of anti-Omp18 antibodies in convalescent-phase sera. The study showed that we could detect anti-Omp18 antibodies with recombinant Omp18-6×His in seven of nine serum samples from patients who were suspected to have suffered from a *C. jejuni* infection. These serum samples gave a positive result in the complement binding reaction with *C. jejuni*. In contrast, among 50 serum samples from healthy human blood donors, we found only 1 serum sample that gave a weak reaction with Omp18-6×His. Because this serum sample also reacted with several other proteins of whole cells of *C. jejuni* on Western blots (immunoblots) (data not shown), we interpret the reaction with Omp18 as evidence of past infection with a campylobacter.

While flagellin and the major outer membrane protein of *C. jejuni* are the predominant antigens recognized by human reconvalescent-phase sera (22), the reactions are diverse and are often restricted to the infecting strain. The reaction of immune and reconvalescent-phase sera with Omp18 is relatively minor, but it is consistently present. The evidence showing the high degree of conservation and the surface exposure of Omp18 therefore make this protein a good candidate antigen for the serodiagnosis of *Campylobacter* infections.

In summary, we have cloned and characterized the gene of an immunogenic outer membrane protein, Omp18, of the reference strain *C. jejuni* ATCC 29428. The protein belongs to the family of bacterial PALs, which are present in all *C. jejuni* strains tested. This is not only of more general fundamental interest, but the Omp18 protein also seems to elicit an immune response in humans infected with *C. jejuni*. Together with the evidence implicating other PALs in immune responses against various gram-negative pathogens, Omp18 is suggested to be a valuable antigen for the development of serodiagnostic assays of *C. jejuni* infections.

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