Purification, characterization and sequence analysis of Omp50, a new porin isolated from *Campylobacter jejuni*

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A novel pore-forming protein identified in *Campylobacter* was purified by ion-exchange chromatography and named Omp50 according to both its molecular mass and its outer membrane localization. We observed a pore-forming ability of Omp50 after re-incorporation into artificial membranes. The protein induced cation-selective channels with major conductance values of 50–60 pS in 1 M NaCl. N-terminal sequencing allowed us to identify the predicted coding sequence Cj1170c from the *Campylobacter jejuni* genome database as the corresponding gene in the NCTC 11168 genome sequence. The gene, designated *omp50*, consists of a 1425 bp open reading frame encoding a deduced 453-amino acid protein with a calculated pI of 5.81 and a molecular mass of 51 169.2 Da. The protein possessed a 20-amino

acid leader sequence. No significant similarity was found between Omp50 and porin protein sequences already determined. Moreover, the protein showed only weak sequence identity with the major outer-membrane protein (MOMP) of *Campylobacter*, correlating with the absence of antigenic cross-reactivity between these two proteins. Omp50 is expressed in *C. jejuni* and *Campylobacter lari* but not in *Campylobacter coli*. The gene, however, was detected in all three species by PCR. According to its conformation and functional properties, the protein would belong to the family of outer-membrane monomeric porins.

Key words: channel-forming properties, conductance.

INTRODUCTION

The Gram-negative bacteria are covered by an outer membrane, which excludes many of the toxic compounds that exist in the external medium. These bacterial cells, however, must exchange molecules with the environment. The porin proteins involved in this function contain large water-filled channels that non-specifically mediate the passive exchange of ions and small hydrophilic molecules. The porins can be divided into two classes: (i) the classical trimeric porins such as OmpF and OmpC in *Escherichia coli* and OprD in *Pseudomonas aeruginosa*, conferring very high permeability, and (ii) the monomeric pore-forming proteins with much lower permeability, such as OmpA in *E. coli* [1–3] and OprF in *P. aeruginosa* [4]. Moreover, although these proteins are essential in physiological processes they also play an important role in the pathogenicity of these organisms [5–7].

In the case of the food-borne human pathogen *Campylobacter jejuni*, only one porin has been identified [8]. This protein has an apparent molecular mass of about 45 kDa [9,10] and belongs to the trimeric porin family [11] with conductance properties similar to the OmpC porin of *E. coli* [12]. By analogy with other well-known Gram-negative bacteria, one can hypothesize that several pore-forming proteins are required to ensure the transport of hydrophilic molecules across the outer membrane of *Campylobacter*. Thus even for *C. jejuni*, which has a relatively small genome [13,14], other porins might be produced with different functional properties to the major porin activity already identified. The level of expression of such porins may depend on culture conditions, thus causing difficulty in identification and characterization.

In previous work, we developed a method for purification of the major outer-membrane protein (MOMP), the major porin of *Campylobacter*, involving outer-membrane preparation followed by specific detergent extraction and chromatography [11]. In this study, to identify poorly expressed porin proteins, we analysed a large amount of outer-membrane detergent extract by ion-exchange chromatography. We report here the identification and characterization of a novel porin protein of *C. jejuni*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

C. jejuni strains 79AH and 85H were described previously [15]. *C. jejuni* strain 81176 was from the Laboratory Center for Disease Control (Winnipeg, Canada). Strains listed below were provided kindly by Gwenolla Ermel (Agence Française de Sécurité Sanitaire des Aliments, Ploufragan, France): there were nine *C. jejuni* (96C6, 96C37, 96C101, 96C399, 96C545, 96C571, 96C579, 96C668 and 96C672); seven *Campylobacter coli* (96C1, 96C12, 96C13, 96C25, 96C47, 96C151 and 96C208) and two *Campylobacter lari* (96C15 and 96C22) strains. All isolates were grown on blood agar plates for 48 h at 37 °C under micro-aerobic conditions.

Protein purification

Bacterial cells were harvested and washed in TE buffer (10 mM Tris/HCl/1 mM EDTA, pH 7.2). After centrifugation, the cell pellet was washed in glycine/HCl (100 mM, pH 2.2) in order to

Abbreviations used: MOMP, major outer-membrane protein; Octyl-POE, *n*-octyl polyoxyethylene.

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Figure 1 Ion-exchange chromatography on outer-membrane protein extracts

Proteins (20 mg) extracted in Octyl-POE (50 ml) were loaded on to the column. After extensive washing the salt gradient was started from 0–1 M NaCl and was monitored by conductivity (right-hand *y* axis and thin line). A_{280} was monitored in milli-absorbance units (mAU; left-hand *y* axis and thick line). The chromatographic volume indicated on the *x* axis begins at the sample injection.

extract associated outer-membrane proteins. The bacterial pellet was washed twice in Tris/HCl (10 mM, pH 7.2) and sonicated in the same buffer. After sonication, cell debris was removed by centrifugation (6000 *g*) for 30 min and the total membrane fraction was recovered by ultracentrifugation (100 000 *g*) for 1 h at 4 °C.

The inner-membrane fraction was solubilized in 10 mM Tris/HCl (pH 7.6) containing sodium lauryl sarcosinate (0.1 %, w/v) [12]. The outer-membrane vesicles were then recovered by ultracentrifugation (100 000 *g*) for 1 h at 4 °C. The supernatant was discarded and the pellet was resuspended in sodium phosphate buffer (20 mM, pH 7.6) containing *n*-octyl polyoxyethylene (Octyl-POE, Bachem AG, Bubendorf, Switzerland) at a 1 % final concentration. This led to the specific recovery of integral outer-membrane proteins into Octyl-POE micelles [11], which were then separated from insoluble material by ultracentrifugation (100 000 *g*) for 1 h at 4 °C. After ion-exchange chromatography, using an Akta explorer on a Mono-Q column (both from Amersham Pharmacia Biotech), the purified protein preparation was stored at –20 °C.

Preparation of antisera and immunogold labelling

Antiserum to peak-1 antigen was raised in rabbits by three successive injections of 100 µg of protein, subcutaneously on day 0 and day 32 and intramuscularly on day 21. Animals were killed on day 40. Bacteria (*C. jejuni* strain 85H) were fixed for 1 h at 4 °C in PBS (150 mM NaCl/3 mM KCl/1 mM KH_2PO_4 /10 mM Na_2HPO_4 , pH 7) containing 2 % (w/v) paraformaldehyde and 0.2 % (v/v) glutaraldehyde. They were dehydrated in dimethyl-formamide and embedded in Lowicryl K4 M. Ultrathin sections were deposited on Formvar carbon-coated grids. Grids were then floated on 5 % BSA in PBS for 10 min to minimize non-specific adsorption. Incubations were performed with rabbit antibodies (1:500 in PBS containing 0.5 % BSA) for 2 h at room temperature and, after several washes with PBS, with gold-coated Protein A (stock solution absorbance, 4 at 520 nm; diluted 30-fold with PBS containing 0.5 % BSA) for 1 h. The grids were washed with water and stained with uranyl acetate for 4 min and lead citrate for 1 min. In control experiments, rabbit non-immunized serum was substituted for the specific antibodies.

Subcellular fractionation

The total membrane fraction was obtained by ultracentrifugation (100 000 *g* for 1 h at 4 °C) of bacterial cells first disrupted by sonication. Outer membranes were isolated after selective extraction of inner membranes from the total membrane pellet, with sarcosyl as described by Filip et al. [16]. Outer-membrane proteins were solubilized further using Octyl-POE as described previously [12]. The methods of Schnaitman [17] were also used to isolate outer membranes by selective solubilization of the inner membranes by Triton-X100 according to Huyer et al. [9]. Samples were recovered by ethanol precipitation prior to electrophoresis.

SDS/PAGE and immunoblotting

Analysis of proteins by SDS/PAGE and immunoblotting were done as described previously [11].

Determination of the N-terminal sequence

The N-terminal amino acid sequence was determined from protein (2 nmol) transferred to PVDF using stepwise Edman degradation on an Applied Biosystems model 470A gas-phase sequencer.

Reconstitution in planar lipid bilayers

Virtually solvent-free planar lipid bilayers were formed by the method of Montal and Muller [18], as modified by Saint et al. [2], using asolectin IV-S (Sigma, St Quentin Fallavier, France). The current fluctuations were recorded using a BLM 120 amplifier (Biologic, Claix, France) and stored on a digital tape recorder (DTR 1202, Biologic). Currents and amplitude histograms were obtained from the stored signals using Satori software from Intracell (Royston, Herts, U.K.). Lipid bilayers were bathed with 1 M NaCl/10 mM Hepes (pH 7.4). Protein was solubilized in Octyl-POE and added to the measurement compartments (4 ml) in a symmetrical manner, typically at 0.25–1 ng/ml or 2.5–10 ng/ml for the single-channel and selectivity experiments respectively. Selectivity experiments required an NaCl gradient of 0.2 M on the *cis* side to 1 M on the *trans* side of the lipid bilayer. The zero-current potential was corrected by deducing the asymmetric potential due to the salt gradient.

Chromosomal DNA isolation

Bacterial cells were harvested following 24 h of growth on Columbia agar plates in TE buffer, and centrifuged. The DNA was prepared according to the method described by Ausubel et al. [19].

PCR assays

The *C. jejuni* MOMP gene (see the Sanger Centre website, http://www.sanger.ac.uk/Projects/C_jejuni/) was amplified using two primers, MOMPC (5'-GCTACTCCACTTGAA-GAAGC-3') and MOMP D (5'-TCGTTGAAAGTATATCC-ACC-3'), which generated a 982 bp amplification fragment from *C. jejuni* strain 85H chromosomal DNA. The gene encoding Omp50 (from the Sanger Centre website) was amplified using primers P50C (5'-TGTAAGCTGAACTTGCC-3') and P50D (5'-GCCGTTCTCTTGTCATTC-3'), which generate a 1100 bp amplification fragment from *C. jejuni* strain 85H. Amplification reactions were performed in a total volume of 50 µl with 0.2 µM of each primer, 100 ng of genomic DNA and 2 units of *Taq* DNA polymerase (Roche) with 100 µM dNTPs and buffer (10 mM Tris/HCl/50 mM KCl, pH 8.3/1.5 mM MgCl₂), as recommended by the manufacturer. After a first denaturation step for 2 min at 94 °C the amplification mixture was subjected to 30 cycles of amplification as follows: samples were incubated for 30 s at 94 °C, for 30 s at 56 °C and for 1 min at 72 °C in a Perkin-Elmer GenAmp PCR system 2400. Samples were analysed by electrophoresis on agarose gels and by ethidium bromide staining.

RESULTS

Purification of the Omp50 protein

The Omp50 protein was first observed as an additional peak during MOMP purification. Based on this property, a method of purification of the protein Omp50 was developed with minor modifications of the MOMP preparation. Given the use of a

Figure 2 SDS/PAGE and immunoblotting

Samples corresponding to peak 1 (lanes 3 and 4) and to MOMP (lanes 1 and 2) were solubilized in sample buffer and treated at either room temperature (lanes 1 and 3) or 96 °C (lanes 2 and 4). (A) Coomassie Brilliant Blue staining; (B) immunoblotting using anti-Omp50 as probe; (C) immunoblotting using anti-MOMP. M, molecular-mass standards, from top to bottom in kDa, 94, 67, 43 and 30.

Figure 3 Subcellular localization of the protein

(A, B) Detergent-solubilized fractions were separated on SDS/PAGE and transferred to nitrocellulose. Lanes 1, total bacterial proteins; lanes 2, total membrane fraction; lanes 3, Triton X-100-soluble fraction; lanes 4, Triton-EDTA-soluble fraction; lanes 5, lauryl sarcosinate extract; lanes 6, Octyl-POE-soluble fraction. (A) Immunoblotting using anti-MOMP antiserum; (B) immunoblotting using anti-Omp50 antiserum. Only the relevant parts of the blots are shown. (Lower panel) Immunogold labelling of *C. jejuni*. Thin sections of Lowicryl-embedded *C. jejuni* (strain 85H) were incubated with specific antiserum raised against the protein isolated in the peak 1. The labelling of the immunocomplex was carried out with gold-coated Protein A (gold particle size, 7 nm).

highly selective outer-membrane protein extraction methodology, this product (Figure 1) was predicted to correspond to a new outer-membrane protein of *Campylobacter*.

Protein identification and conformational arrangement

The protein profile of peak 1 was determined using SDS/PAGE to contain a major band with a molecular mass of 50 kDa (Figure 2A, lane 4), which we named Omp50. The sample corresponding to peak 1 was solubilized in 3% SDS at either room temperature or 96 °C to study the folding stability of this protein. Even with the high SDS concentration the protein was observed to have a smaller relative molecular mass of 35 kDa (Figure 2A, lane 3). Heating at 96 °C was necessary to ensure complete disruption of the secondary structure, allowing full denaturation of the protein and subsequent migration at a molecular mass of 50 kDa (Figure 2A, lane 4). Moreover, no oligomer was observed under the conditions that were used

Figure 4 Pore-forming activity of *C. jejuni* Omp50

Single-channel current recordings of Omp50 during two different experiments. The applied potential was 80 mV (electrolyte, 1 M NaCl buffer). The associated histogram measures a single-channel conductance level of $G = 55 \pm 3$ pS. Digitization rate, 3000 Hz; filter, 300 Hz. O, open state; C, closed state.

previously to recover the trimeric form of MOMP (results not shown).

Antigenic relationship between Omp50 and MOMP

Given the selective purification scheme and conformational similarities between Omp50 and MOMP, the antigenic cross-reactivity of the two proteins was investigated. A rabbit antiserum was prepared using Omp50 as the immunogen. As shown in Figure 2(B), lanes 3 and 4, this antiserum recognized the two conformations of Omp50, whereas MOMP was not detected (Figure 2B, lanes 1 and 2). Conversely, anti-MOMP antiserum (described in [20]) did not recognize either of the Omp50 forms (Figure 2C, lane 3 and 4). Thus no antigenic cross-reactivity was observed between Omp50 and MOMP.

Cellular localization of the protein

In both methods used to perform the selective extraction of total membrane fraction, Triton X-100/Triton-EDTA or lauryl sarcosinate/Octyl-POE, Omp50 was co-localized with the MOMP porin (Figures 3A and 3B). The anti-Omp50 antiserum was used to perform immunogold-labelling of thin sections of *C. jejuni* strain 85H. The specific labelling (Figure 3) localized Omp50 to the outer membrane. The low labelling intensity of Omp50, compared with that of MOMP [20], correlates with the lower amount of Omp50 found in the outer-membrane fraction.

Pore-forming activity of Omp50

Outer-membrane proteins showing such a characteristic conformational behaviour generally belong to pore-forming proteins of Gram-negative bacteria. Therefore, the ability of Omp50 to exhibit channel properties in artificial membranes was studied. Re-incorporation into asolectin bilayers was performed after application of a potential of 80 mV. This protein induced current

fluctuations corresponding to a major single channel conductance of 55 ± 3 pS (Figure 4). Other minor conductance increments of 100, 150–180, 200–230 and 300 pS, which could correspond to aggregate insertion in the bilayers, were also observed (results not shown). We did not observe the stepwise increase of current fluctuation, usually observed for porins like OmpF or OmpC from *E. coli* [21] and MOMP from *Campylobacter* [12]. No voltage gating in the ± 160 mV voltage range was detected. After establishing an NaCl concentration gradient (0.2–1 M, *cis-trans*), a zero-current potential value of +13 mV (after deducting the asymmetric potential) was found. According to the Goldman–Hodgkin–Katz equation [22], the calculated P_{Na}/P_{Cl} ratio of 2.3 indicated slight cationic selectivity for the Omp50 porin.

Sequence of the porin Omp50

To further characterize Omp50 and identify the gene encoding this protein in the *C. jejuni* NCTC 11168 genome, the N-terminal amino acid sequence was determined to be KNTDDEITKL-QKQLAQIQAELAQIRKERE and was compared with deduced amino acid sequences derived from the *C. jejuni* genome database (http://www.sanger.ac.uk/Projects/C_jejuni/). We found that the putative coding sequence Cj1170c contained these 30 residues (Figure 5). Conversely, searches of the protein and gene databases failed to reveal any similarity to other known proteins. The N-terminal amino acid sequence suggested that the cleavage site for the signal sequence is localized between Ala-48 and Lys-49 of the Omp50 open reading frame. Three initiating codons could be used, corresponding to methionine residues 1, 27 or 29, but a potential [23] ribosome-binding site (AGGA) ends nine bases upstream from the third ATG codon. Thus using the third ATG codon, the signal peptide is 20 residues long. Then Met-29 is followed by two positively charged residues (Lys-30 and Arg-32) and a stretch of 13 residues containing short lateral chains (from Ser-34 to Leu-46), a structure in accordance with the consensus signal sequence [24]. The mature protein has a calculated molecular mass of 51169.2 Da, which is in agreement with the apparent migration of the protein on SDS/PAGE (Figures 2A and 2B, lanes 4). The calculated isoelectric point of the mature protein is 5.81, which is 1.3 pH units higher than MOMP but, like many Gram-negative porins, is still acidic. The characteristic C-terminal Phe residue is found in the sequence of Omp50 (Figure 5). Only weak similarities were found with MOMP, supporting the absence of immuno cross-reactivity between the two proteins (Figures 2B and 2C).

Distribution of Omp50 among species and strains

To determine whether Omp50 is a common porin of the genus *Campylobacter*, we examined the distribution of the Omp50 antigen in various species. The antiserum prepared in this study was used to probe samples of total proteins prepared from 21 isolates of *Campylobacter* by Western-blot analysis. In order to compare the expression of MOMP and Omp50, the experiments were done in duplicate and immuno-stained with the respective antiserum. Whereas MOMP was expressed in all the isolates tested (Figure 6), Omp50 was not expressed by *C. coli*, but was expressed in *C. jejuni* and *C. lari* (Figure 6). The *omp50*-specific primers (see Figure 5 and the Materials and methods section), and MOMP primers as a control (see the Materials and methods section), were then used in PCR experiments to determine whether *C. coli* contained the gene *omp50*. As shown in Figure 6, the gene coding for Omp50 was detected from all but one of the

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1/1                               31/2
ATA AGT TTG ACT AGC TAA AAA TGA TTA ATG TAT AAT TTT TTT TTA AAC TTT TTT TTC TAT
----- m y n f f l n f f f y

61/12                               91/22
AAT CAC AAT TTT AAT TTG CTA AAT GTA ACA AAA TTA CAA GGA GTT ATG ATT ATG AAA ACT
n h n f n l l n v t k l q g v m i M k t

121/32                               151/42
AGG TTT TCA CTT ATT TTA AGT GCT TGT CTT CTT TCA TCT TCA CTT TTT GCT AAA AAT ACA
r f s l i l s a c l l s s s l f a K N T

181/52                               211/62
GAT GAT GAG ATA ACT AAG CTT CAA AAA CAG CTT GCA CAA ATT CAA GCT GAA CTT GCA CAA
D D E I T K L Q K Q L A Q I Q A E L A Q

241/72                               271/82
ATC AGA AAA GAA AGA GAA GCT CAA GTT AAA CAA AAT GAA GCT GTA AAA GCT GAA CTT GCC
I R K E R E a q v k q n e a v k a e l a
.....
.....
.....
CCA AGT ATC AAT GAT CCG CTT AAT ATA AGA ATG ACA AGA GGA ACG GCA CAT GAT TTT TAT
p s i n d p l n i r m t r g t a h d f y

1381/452                               1411/462
GTG ATT TAT CAA CTT GAT AGA TAT CAA TTC TTA CGC TTA AGC TAC ACC AAT ATC CAA AAC
v i y q l d r y q f l r l s y t n i q n

1441/472                               1471/482
ATT TGG GGT AAT CGT GGC TTA CCA TTT GGT GGA GCG AAA AAA GAT AAA GCA AGA GCT GAT
i w g n r g l p f g g a k k d k a r a d

1501/492                               1531/502
AAT ATC ATG TTA ATG TAT AAT GTA AAA TTC TAA GGA GCA AAA GAT GAG AAA AAT ACT TTT
n i m l m y n v k F STOP

1561                               1591
TGC AAG TTT ATT ACC TAT CAT TGC TTT GGC TGA TTG TGC

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Figure 5 Sequence analysis of the NCTC 11168 strain Omp50 coding gene

These sequence data were produced by the *C. jejuni* Sequencing Group at the Sanger Centre and can be obtained from <ftp://ftp.sanger.ac.uk/pub/pathogens/cj/>. The putative ribosome-binding site (AGGA) is underlined; the putative first methionine is in bold; amino acid residues identified by sequencing of the purified protein are in upper-case letters; the arrowhead indicates the cleavage site between the signal sequence and the mature protein. The two arrows show the positions of the oligonucleotides used in the PCR experiments.

strains tested. These data suggested that, although most strains of *C. jejuni*, *C. coli* and *C. lari* contain *omp50*, the gene may not be expressed in *C. coli* strains. Alternatively, the gene product may be not processed or folded correctly and subsequently degraded in these strains.

DISCUSSION

The outer-membrane components of a Gram-negative bacterium play an important role in host-parasite interaction. However, little is known at the molecular level about the outer-membrane proteins of *Campylobacter* species and their respective physiological functions. Previously we studied the biochemical and biophysical properties of the *C. jejuni* MOMP protein [11,12,20]. In the present work, we investigated the properties of another component of the *Campylobacter* outer membrane.

The protein Omp50 was isolated after selective extraction of proteins from outer-membrane vesicles followed by ion-exchange chromatography. We showed that Omp50 has a stable folded monomeric conformation that can only be denatured by high temperature in the presence of detergent. The deduced amino acid

sequence of the protein indicates that Omp50 contains a cleavable signal sequence and a phenylalanyl residue at the C-terminus characteristic of outer-membrane protein localization [25]. Taken together, these results suggest strongly that this protein belongs to the group of monomeric porins illustrated by the OmpA-like family [2,3]. Although a motif, XPXP, in the sequence is similar to the consensus XPXPXP described in OprF [26] and to XPXPXPXP in OmpA [27], the similarity with the sequence of the monomeric porin class is weak.

Omp50 is able to induce ion channels in artificial lipid bilayers with conductance values of 50–60 pS in 1 M NaCl. These values are markedly smaller than those of trimeric porins [12,21]. Numerous monomeric porins, however, display low single-channel conductance, including porins of the Hop family from *Helicobacter pylori* (240–360 pS in 1 M KCl) [28] and the major porin OprF from *P. aeruginosa* (340 pS in 1 M KCl) [4] or *P. fluorescens* (250 pS in 1 M NaCl) [29]. However, with such a low conductance value of 55 pS, Omp50 could belong to the family of channels with substrate specificity. For the *E. coli* porin Tsx, a single-channel conductance value of 10 pS in 1 M KCl and a nucleoside specificity were detected [30] and the porin OprB

Figure 6 Immunoblotting and PCR assays

Immunoblotting was carried out on samples of total bacterial proteins using either anti-MOMP or anti-Omp50 as indicated. C.j, C.c and C.l are for *C. jejuni*, *coli* and *lari* isolates, respectively; strains numbers are indicated. PCR with either MOMP and MOMP or Omp50C and Omp50D were analysed by electrophoresis on 1% agarose gel. Only the relevant parts of the agarose gels and blots are shown.

from *P. aeruginosa* has a conductance value of 25 pS in 1 M KCl and a glucose-binding site [31]. In other respects, the moderate cation selectivity ($P_{Na}/P_{Cl} = 2.3$) and the lack of voltage sensitivity make Omp50 pores distinct from the general diffusion pores observed with *E. coli* OmpF or OmpC ($P_{Na}/P_{Cl} = 4.5$ and $P_K/P_{Cl} = 13.4$, for OmpF and OmpC respectively) [32,33].

Prior to this report, MOMP was the only outer-membrane pore protein functionally characterized in *Campylobacter*. Now, the Omp50 appears to constitute an alternate pore-forming protein class that is expressed at lower levels in *C. jejuni* and not detected in *C. coli*. Therefore the identification and characterization of Omp50 will open a new area of research in the field of outer-membrane permeability and in the study of bacteria–host interactions for the food-borne pathogen *Campylobacter*.

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