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MOMP, a Divergent Porin from *Campylobacter:* Cloning and Primary Structural Characterization

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We report a structural analysis at the molecular level of MOMP from *Campylobacter,* a gram-negative bacteria responsible for diarrhea. The corresponding gene was cloned and sequenced. Sequence comparison of seven MOMP sequences (three extracted from protein databases and four determined in this study) from distinct strains indicated alternation of preserved and divergent regions. No other significant sequence similarities could be detected. Comparison of MOMP with the crystal structures of other porins strongly suggested that it might adopt a similar fold and revealed the conservation of the monomer–monomer interface. The conservation clustered in the regions comprising or interacting with the loop L2. On the contrary, strands not involved in the interface are more divergent. Proteolysis assays and biochemical treatment supported the proposed model. Our study suggested that MOMP belong to the maltoporin super-family sharing common structural motifs. In view of this model we discuss its specificity and its global stability.

Key Words: **porin; gene cloning; membrane protein; molecular modeling; structure–function relationship; pathogenicity.**

Campylobacter jejuni is recognized as an important agent of gastroenteritis through the world. Campylobacter infection is usually, a mild to moderate self-limiting illness. However, patients with severe enteritis should usually be treated, with erythromycin and tetracycline (1). The increasing level of resistance to these molecules has encouraged the screening of other molecules, which could be used as potentially alternative drugs. The outer membrane of such a, Gram negative bacteria, is a physico-chemical barrier against hydrophilic compounds. Although porins contribute to the diffusion of small hydrophilic solutes, these pore-forming proteins exhibit different selectivity to ions or molecules. Consequently, the impermeability has been evoked, to explain the resistance of *Campylobacter* against various classes of antibiotics (2).

In *Campylobacter* two porins are identified to date, named Major Outer Membrane Protein (MOMP) (3) and Omp50 (4). The MOMP is important in envelope structure of the bacteria (5), putative vaccine component (6) and for its proposed role in the pathological process (7, 8). The well-studied MOMP has an apparent molecular weight of around 45 kDa (9). This porin was shown to exhibit peculiar properties, the monomer and the trimer appearing as stable conformations which were both able to induce pore forming activity in vitro (10, 11). Various structures of all-β transmembrane proteins were recently solved ranging from the small monomeric eight-stranded OmpA (12) to the larger monomeric twenty-two-stranded siderophore transporter FhuA (13) and including the well-studied trimeric sixteen- and eighteen-stranded porins (14, 15). The latter sub-family contains the sucrose and maltose specific channels.

The particular behavior of this porin and its putative function in virulence (7, 8), prompted us to analyze its sequence. The first protein sequence database searches using PSI-BLAST indicated no significant similarity between the *Campylobacter* MOMP and other porins. While straightforward in cases of high sequence identity (over 35%), alignment of all the sequences belonging to the same structural family (16) becomes more hazardous and tedious at low sequence identity level (below 25%) (17). Additional methods are needed to correctly gather and align all the sequences for the analysis of a new protein sequence distantly related to a given structural super-family (18, 19). The better

Abbreviations used: aa, amino acid; HCA, hydrophobic cluster analysis; MOMP, major outer membrane protein.

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conservation of the three-dimensional structures may lead to the identification of a distantly related homologue from an already determined three-dimensional structure (20). Careful analysis of the deduced structural model was shown to be helpful for the determination of specific functional features in spite of the low sequence identity (21, 22).

The structure of MOMP was investigated using both a comparative molecular modeling, proteolytic assays with outer membrane embedded proteins, or chemical treatment of the purified protein. The model highlighted the putative role of few amino-acids either in the function or the structure of the porin. Statistical significance of these sequence motifs was checked using short sequence pattern searches. Our analysis suggested that MOMP from *C. jejuni* belongs to the trimeric maltoporin super-family (23).

MATERIALS AND METHODS

Bacterial strains, plasmid and growth conditions. The *Campylobacter jejuni* strains 85H and 79AH were previously described (33). The *C. coli* strain 96C1 and 96C12 were kindly provided by Gwenolla Ermel (AFSSA-Ploufragan, France). Stock cultures maintained at −80°C were rapidly thawed and grown for 48 h on Columbia agar at 42°C under microaerobic conditions. *Escherichia coli* strain TG1 was used in every DNA manipulation. Plasmid pTAg was purchased from (Ligator, Ingenius) and was used to clone PCR amplified fragments according to the manufacturer.

Molecular cloning. Probe labeling, DNA hybridization, restriction analyses, Southern transfer and colony blotting were performed using standard procedures as previously described (33). The nucleotide sequencing, was performed by Eurogentec (Belgium). PCR was done at 50 cycles of denaturation 1 min at 94°C, hybridization 2 min at 42°C, elongation 2 min at 63°C with a terminal elongation of 10 min at 72°C.

Proteinase K treatment of bacteria. Bacteria harvested from plates were washed in 10 mM Tris, 10 mM EDTA (pH 8.0) and further suspended in 10 mM Tris (pH 8.0; OD 600 nm = 0.5) and treated with proteinase K (Roche-Boehringer Mannheim) at 25 μg/ml final concentration. Incubations were carried out at 37°C and, after the additions of Pefabloc (Roche-Boehringer Mannheim) at 1 mM final concentration, aliquots were precipitated by the addition of Tri-Chloro Acetic acid 15% final concentration prior to electrophoresis.

SDS–PAGE, immunoblotting and protein purification. Analysis of proteins by SDS–PAGE and immunoblotting were done as previously described (10). The two specific antisera directed against MOMP and Omp50 respectively were described elsewhere (4, 28). The previously reported purification (11) was simplified by omitting the chromatofocusing step.

Sequence comparison and molecular modeling. Protein sequence database searches were performed with the program PROPSEARCH (25), the PSI-BLAST version 2.0.5-program (16) with default parameters and with PHI-BLAST program (29) using short conserved signature deduced from multiple alignments or porin structure analysis. The same motifs were used to screen databases with the program PATTINPROT (30) and further refined. The final signature used in PATTINPROT comprised the following motif [PVILFAMY]-X-[VILFMYW]-R-[QE]-[VILFAM]-X-[VILFAM]-X-[GVILFAMY]-X-[NG]-X(3,9)-[AG]-[AST]-[VST]-[VILFACMWY](2)-A-G-K-[QR]-X(13,18)-[TVILFACMWY]-X-G-[PSTR]-G-[GVIAC]-X-[GVILFACMWY]-X(4)-[GVILFACMWY]. An additional specific motif [STVND]-X(2)-[VILFACMWY]-Q-[VILFACMWY]-X(3)-[FYW] comprises the very C-terminus end. Pairwise and multiple alignments were performed using Hydrophobic Cluster Analysis as previously described (18), in order to delineate the structurally conserved regions along the amino acid sequences. Topology predictions were performed using a neural network based server (26). Alignments were subsequently refined using the program TITO (20) using the known SPR and MPR crystal structure (14, 15). The porin secondary structures (mainly β-strand) were assigned during TITO processing and the secondary structure derived by homology for MOMP were used as additional restraints in the following modeling steps. Three-dimensional models were built using as a combined template SPR and MPR structures and the program MODELLER 4.0 (34) and were assessed using Verify3D (35). These three-dimensional structures were visualized on a UNIX workstation using XmMol (36). The residue numbering correspond to the position in the mature MOMP from *C. jejuni* 85H.

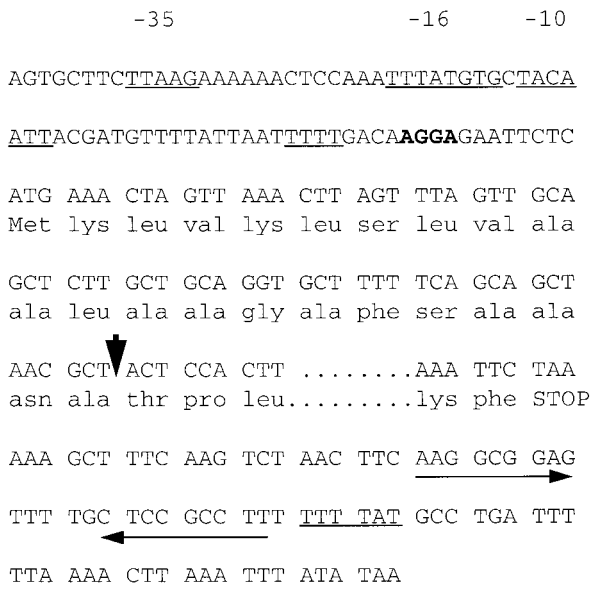

FIG. 1. Gene structure. The putative ribosome binding site (AGGA) is bold type; the arrowhead indicates the cleavage site between the signal sequence and the mature protein. The sequences homologous to the consensus sequence for *C. jejuni* promoters (37) are underlined. Arrows indicate the sequences corresponding to the stem loop structure of the putative transcription terminator.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of the MOMP Locus

The N-terminal sequence of MOMP isolated from strain 85H was previously determined (10). This sequence and the sequence corresponding to the MOMP-OmpC common epitope C3 (10) were reverse translated and the corresponding degenerated oligonucleotides were used in a PCR assay using the 85H chromosomal DNA as template. Among the three products obtained (data not shown), one fragment gave a sequence corresponding the amino-terminal sequence and was further used as probe on Southern hybridization. Two *Hind*III fragments of 720 and 657 bp respectively, were cloned and sequenced. They corresponded to the pro-

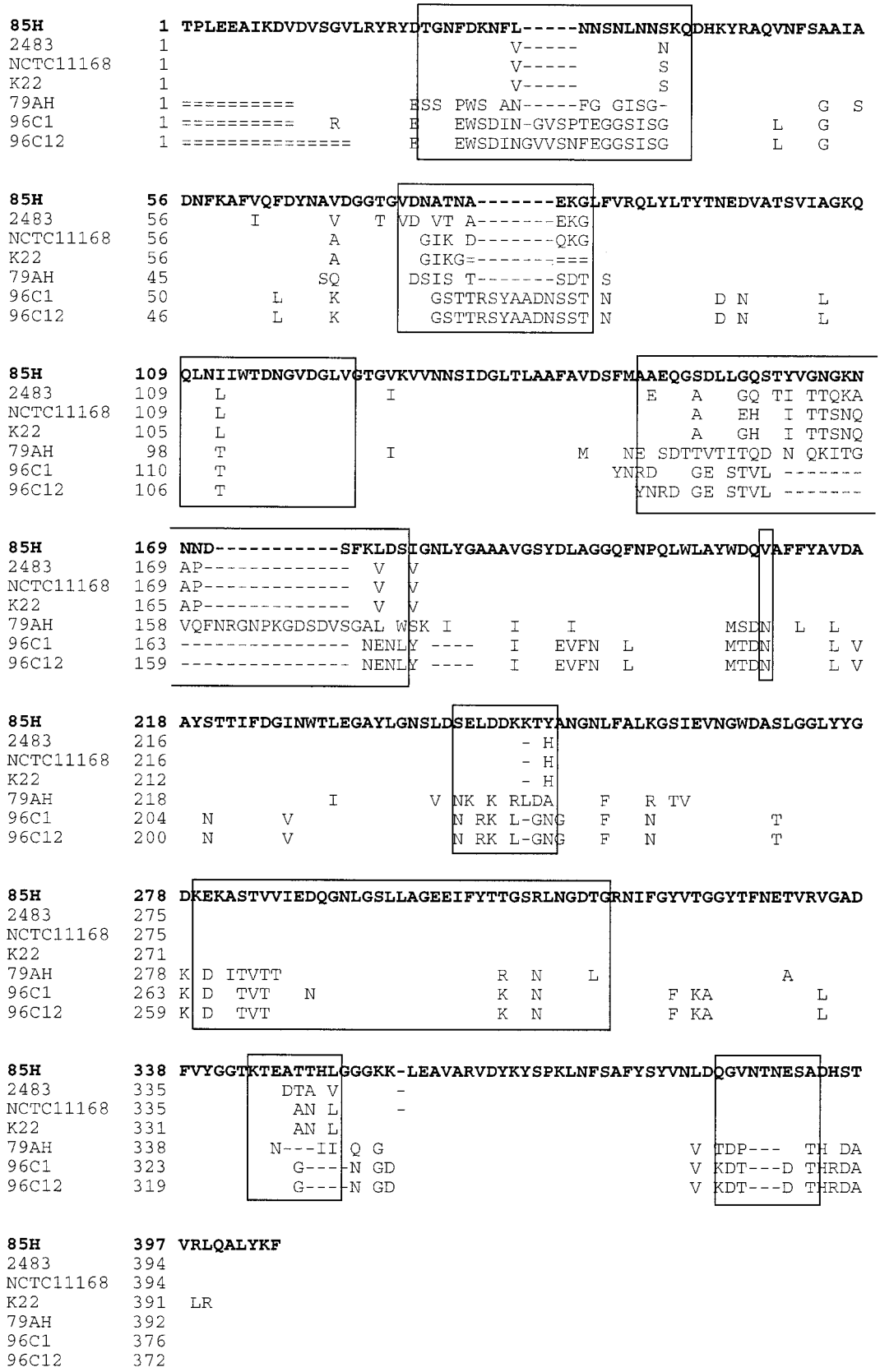

FIG. 2. Sequence comparison of MOMPs. The deduced amino acids sequence of the MOMP gene from strain 85H is presented in bold type. Only the amino acid residues divergent from the 85H MOMP sequence are presented for the other proteins. The name of each sequence corresponds to the name of the strain (2483 GenBank sequence data base Accession No. U96452; NCTC11168 and K22, Swiss Prot protein sequence data base Accession Nos. 89PN38 and P80672); the three other sequences were determined in this study. Dashes indicate gaps in the sequences and double dashes indicate unavailable sequences. Putative external loops determined by the three-dimensional modeling (see Fig. 4) are boxed. Numbers on the left indicate amino acid residue position in the mature proteins.

moter part and the 5′ moiety of the *momp* gene respectively. Southern blot analysis was then performed, using the 657 bp fragment as probe and a 929 bp *Pst*I-*Nsi*I fragment was identified and cloned. Its sequence encompassed the C-terminal moiety of the protein coding sequence. The structure of the MOMP gene is presented in Fig. 1. A single open reading frame of 1281 nucleotides was identified in the fragment. A potential ribosome binding site (24), AGGA, ended 9 bases upstream from the ATG codon. The down-stream flanking region of the gene showed a palindromic sequence with complementarity for 18 of 23 bp (see Fig. 1) which could form a stem loop structure (ΔG = 12.6 kcal/mol) expected for a *rho* independent prokaryotic transcription terminator. The N-terminal sequence of the purified protein corresponded to residues 23 to 55, thus the protein contained a 22 amino-acids signal peptide according to the amino-terminal sequence previously described (10). The processed protein had a molecular weight of 43,898 Da, which agreed well with the estimate of 45,000 Da determined by SDS–PAGE (10) and that measured by ESI experiment (43,837 ± 36).

Sequence Comparison of MOMP from Various Campylobacter Strains

Seven complete or partial sequences of MOMP from various *C. jejuni* strains were compared. Three were previously published (K-22, (8); NCTC 11168, Sanger centre; 2483, Bacon *et al.*, 1997, direct submission) the four remaining were sequenced in this work and named 79AH, 85H, 96C1 and 96C12 according to the name of the corresponding strain. Despite an overall very high level of identity, some variations were observed that clusters in few regions (Fig. 2). Based on this analysis, two sub-groups may be described. One group comprised 79AH, 96C1 and 96C12 harboring larger variations in the primary structure than the second group including the other sequences with only minor amino-acid substitutions and one deletion. This sequence variation was used for structural prediction (23) and the insertion/deletions were attributed to cell-surface exposed regions. According to this hypothesis they would correspond to the external loops L1, L2 and L4, respectively. The lack of further divergence among this 405 aa long MOMP make the other loops and consequently the trans-membrane strands hardly predictable. Sequence database screening using PROPSEARCH (25) revealed some amino-acid composition bias similar to that of the sixteen-stranded porins while the neural network dedicated to outer membrane β-strand protein topology (26) predicted more than 9 extracellular loops (Fig. 3). On the contrary, PSI-BLAST searches in different databanks, using the various *C. jejuni* MOMP sequences as queries, converged immediately in absence of significant similarities with other proteins. However, the full-length sequence of MOMP from various *C. jejuni* strains indicated that their very C-terminus residue is a conserved aromatic residue as in all other porin β-barrels (27).

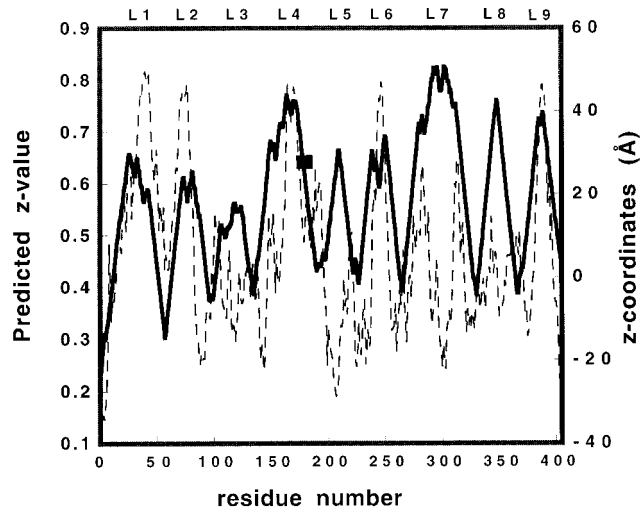

FIG. 3. Predicted and modeled topology of the MOMP. The topology plot of the MOMP from *C. jejuni* 85H is shown as predicted by neural network (http://strucbio.biologie.uni-konstanz.de/~kay/om_topo_predict2a.html; thin dotted line) and as modeled by our approach (solid line). Outer loops are labeled according to the porin topology (L1 to L9). The x-axis corresponds to the residue number while the y-axis corresponds to the z-value predicted by the neural network or the coordinates (of the Cα carbon of each residue) along the z-axis (normal to the membrane surface) in the 3D model presented here. The proteinase K cleavage site in MOMP 85H was predicted by similarity with the observed cleavage site by Schröder and Moser (8) and is indicated by a black square.

Sequence Comparison of Campylobacter MOMP with Other Known Porins

The knowledge of the channel properties of the MOMP, its mainly β secondary structure (10, 11) as well as its trimeric quaternary structure as observed by electronic microscopy (28) prompted us to compare its sequence with known 16 and 18 β-stranded porins.

Sequence alignment of MOMP with known porins using Hydrophobic Cluster Analysis (18) pointed out conservation of the secondary structure and of the overall fold as well as of residues critical for putatively substrate binding and monomer-monomer interface. The 405 aa long MOMP sequence better aligned with 18-strand containing porins rather than with shorter 16-strand containing porins (Fig. 4). The conservation of a similar fold in MOMP was further analyzed through molecular modeling using the sugar-complexed structures of the sucroporin (SPR) and the maltoporin (MPR) (ref 14; code PDB1A0T and ref 15; code PDB2MPR) despite the low sequence identity.

The motif AGK on strand β-5 appeared strictly conserved among MOMP, SPR and MPR. Its structural

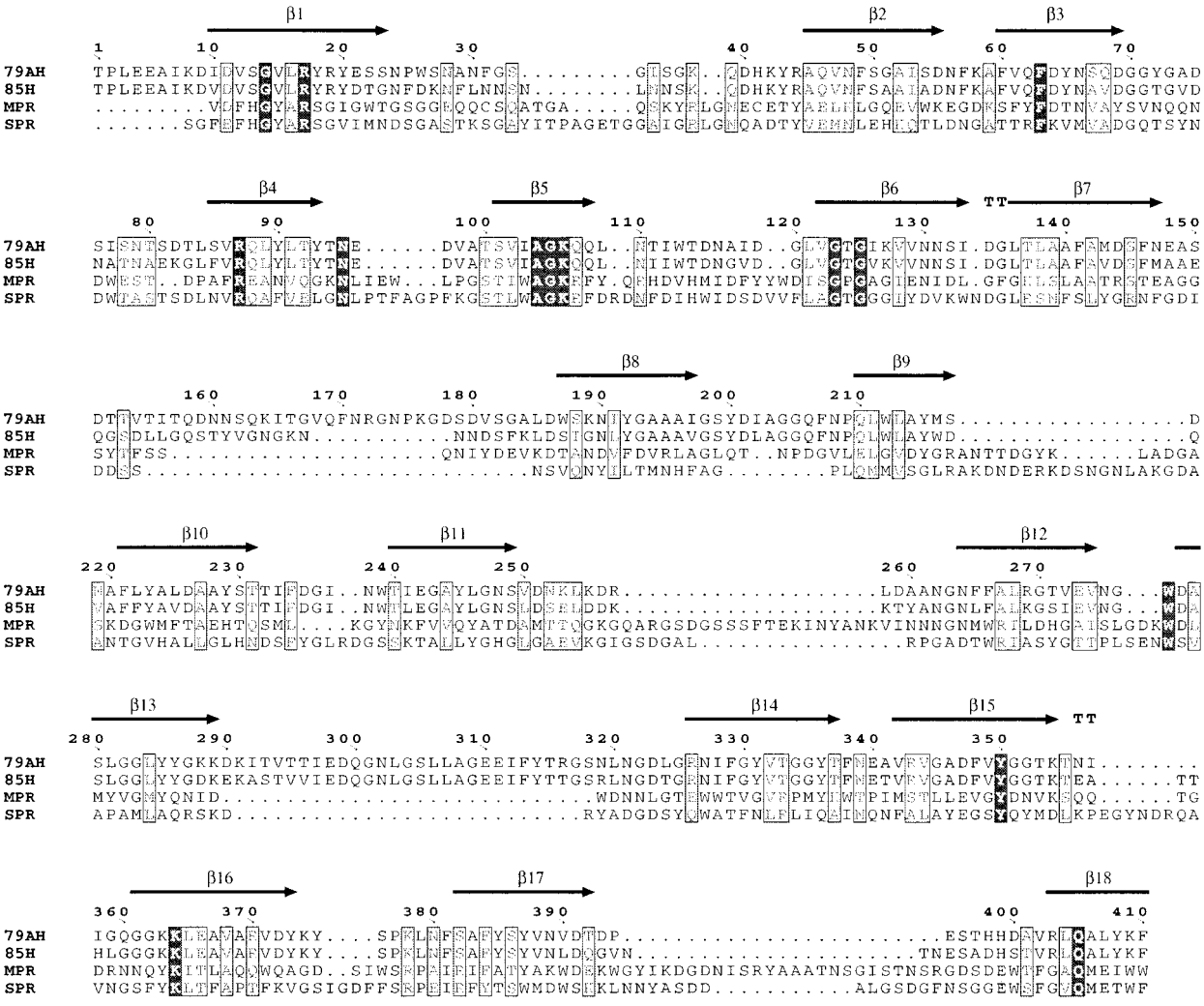

FIG. 4. Sequence alignment: MPR, SPR, 85H, and 79AH. The sequence/structure alignment of the two sequences of MOMP from *C. jejuni* strains 85H and 79AH with the two X-ray structures of MPR (PDB2MPR) and SPR (PDB1A0T). The β-strands assigned on the modeled MOMP are labeled following the porin topology. Similarities are in boxes while strictly conserved residues are in black boxes. The figure was done using ESPript (38).

role at the trimeric maltoporin interface (15) suggested this short sequence might be a signature. The buried lysine side chain from one monomer interact with various carbonyl group of the loop L2 backbone protruding from another monomer into the central pore (Fig. 5). The significance of this motif was check using PHI-BLAST (29) and PATTINPROT (30). Database screening using MOMP sequence and the motif st//AGK (where '/' correspond to any hydrophobic residue, upper and lower case letter to strictly and not strictly conserved residues, respectively) indicated a hit with both MPR and SPR. However, the overall probability score was still high 0.11. Additional motifs (/d/sG//Rxx/ and txx/Q/xxxf) were defined on two other β-strands (β-1 and β-18 in MPR and SPR) lying at the trimer interface. The latter motif comprised the aromatic residues conserved at the very C-terminus end in all the porins. The former corresponded to the N-terminal β-strand, which comprised a β-bulge likely stabilized by the conserved glycine. Conservation of this motif is correlated to that of the β-18 signature as the β-bulge conformation accommodates the bulky side chain of the conserved aromatic residue at the very C-terminus. In addition, a strictly conserved residue of this motif (R17) points toward the central pore and interact with the bound sugar in MPR and SPR (14, 15). Further strand motifs (/x/Rq/x/xxxn and /xGtG/x/xxxx/) were identified corresponding to two other β-strands (β-4 and β-6) lying at the trimer interface. The former strand contributed to two well-conserved charged residues to the central pore, R88 and Q89 in 85H. The equivalent residues in SPR, R90 and Q91 (in MPR: R106 and E107) are in direct contact with the bound sugar (14, 15).

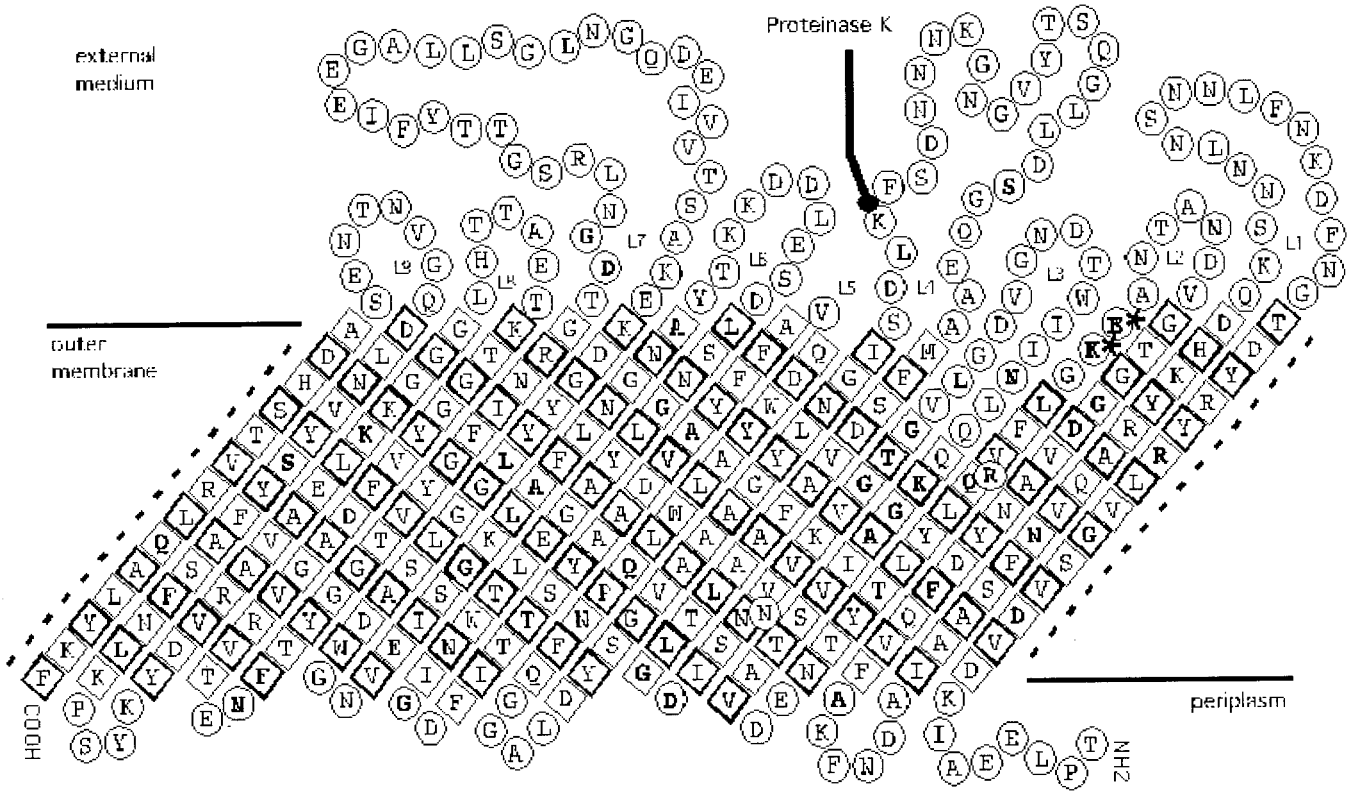

FIG. 5. Schematic overview of the modeled 3D structure. A 2D representation of the 18-strand model of the MOMP from *C. jejuni* 85H is shown embedded in the outer membrane. The interaction between β-strands β1 and β18 is shown as a dotted line. Outer loops are labeled according to the porin topology. Strictly conserved residues between MOMP and SPR are in bold. Residues assigned in loops (or β-bulges) are in circles while residues in internal β-strands are in boxes. Thicker boxes surround residues facing the lipids. The proteinase K cleavage site, in MOMP 85H, is indicated by the black dot and was predicted by similarity with the observed cleavage site by Schröder and Moser (8). Two stars indicate the two residues E82 and K83 in MOMP 85H, substituted in MOMP 79AH toward S81 and D82 and predicted to be responsible for the difference in EDTA susceptibility of these two MOMP quaternary structure.

To improve the statistical significance of this pattern searches a longer motif sequence was determined on a stepwise fashion comprising the short motifs of strand β-4, β-5 and β-6. The expectation value for this final signature was e^{-18} far below the probability score threshold usually accepted (0.00001). The porins containing only 16 strands possess distinct, despite distantly related motifs (gx/x/Gr; gG//x/ and ///G/xyqf) for the β-strands lying at the monomer-monomer interface (β-5, β-6 and β-16 corresponding to β-5, β-6 and β-18 in MPR and SPR).

The MOMP is only 15% identical to sucroporin (SPR) and 13% to maltoporin (MPR) in our final alignment. This difference is made significant by the positions in the structure of the amino-acids conserved in MOMP and SPR but not in MPR. This set comprises mainly residues at the interface such as aspartate 70 (tyrosine in MPR), leucine 85 (proline in MPR) and threonine 125 (proline in MPR). Other strictly conserved residues point into the protein central pore. Some of them interact with the sugar compounds present in the sucroporin or maltoporin complexed structures (14, 15).

Comparative Proteolysis and EDTA Susceptibility

According to our predicted model (Fig. 6), several discrepancies in the sequences may be associated for several divergences in the biochemical and biophysical properties of the MOMPs.

We first analyzed the proteinase K sensitivity of the proteins in intact cells (Fig. 6A and B). Entire bacterial cells were incubated with the protease in the same conditions of temperature and concentration. MOMP from 79AH showed no cleavage during the 4 h of incubation (Fig. 6; Panel A). In contrast, the MOMP from 85H was rapidly cleaved into two major fragments indicated by arrowheads, corresponding to 30 and 15 kDa respectively (Fig. 6; panel B). At the same time the amount of MOMP decreased gradually. A similar pattern was reported with a *C. jejuni* MOMP (8), and N-terminal sequencing identified the proteolytic cleavage at the residue F167. This position is conserved in 85H but not in 79AH and would correspond to the predicted external loop L4 in our model (Fig. 5).

The MOMP from 79AH was selectively extracted and purified according to the method used for 85H (see the

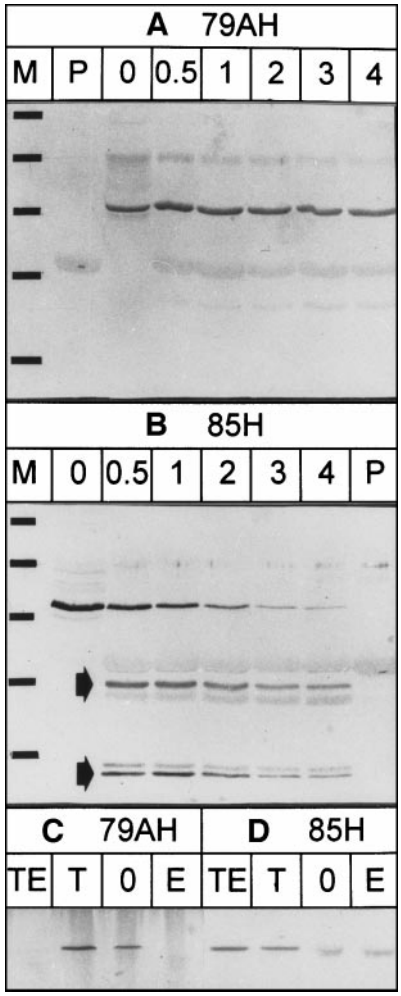

FIG. 6. Proteolysis and comparative electrophoresis. (A and B) Aliquots were recovered from bacterial cell suspension treated with proteinase K, after 0, 0.5, 1, 2, 3, 4 h of incubation at 37°C. Samples were then analyzed by Western blotting using an anti-MOMP polyclonal antiserum. P indicates a proteinase K sample used as control. (C and D) Purified MOMP from Strains 79AH (C) and 85H (D) were incubated with Triton X-100 and EDTA (TE) Triton X-100 (T), water (O), or EDTA (E) prior to electrophoresis in nondenaturing conditions. Only the relevant parts of the gels corresponding to the apparent migration of the trimers are shown.

Materials and Methods section for details). Proteins were diluted alternatively into 1% Triton X-100, 1% Triton-EDTA 5 mM, EDTA 5 mM or water as a control. Electrophoresis were performed in conditions (0.1% SDS) preserving the quaternary structure (10, 28). As shown in Fig. 6C the MOMP isolated from 79AH exhibited no trimeric form in the presence of EDTA even in the presence of Triton X-100, suggesting that a divalent cation may be involved in the trimeric structure stabilization. In contrast, the 85H porin, appeared as a trimer of comparable amount in the presence or absence of EDTA (Fig. 6D). This difference in EDTA sensitivity might correlate with two residue substitutions at the trimeric interface. In 79AH the residues S81 and D82 replaced the residues E82 and K83 (labeled with a star in Fig. 5). The latter two are globally neutral. On the contrary, from each MOMP 79AH monomer, the negatively charged aspartate D82 would point toward the three-fold axis. This result suggested that a cation might nicely fit in as in MPR (calcium binding by the aspartate D78).

In conclusion, a three-dimensional structural homology model for *Campylobacter* porins was generated from the sucroporin and the maltoporin structures and the *Campylobacter jejuni* MOMP sequence. The *Campylobacter* sequence was readily assembled into the 18 β-barrel fold characteristic of both, sucroporin and maltoporin despite low sequence identity. This global fold prediction and the deduced trimeric organization is in agreement with a previous structural analysis of the *C. jejuni* outer membrane (31). Analysis of the trimer interface observed in the SPR and MPR crystal structures suggested that MOMP would form a similar trimer despite its higher tendency to monomerize in presence of SDS (10). The β-strands at the interface are better conserved while those facing the membrane are much less conserved, making the alignment more hazardous in those regions, especially strands β-8 to β-14. This is in agreement with the results of the structural analysis previously described on trimeric porins (32). The modeled interface supported the observed dependence of 79AH trimer to EDTA, while the 85H one is insensitive to EDTA. Similarly, the proteinase K treatment confirmed the proposed topology as well as indicating a simple enzymatic test to characterize MOMP from newly isolated *Campylobacter* strains.

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Note added in proof. 22 new sequences of MOMP from various *C. jejuni* strains were recently published (Zhang, Q., Meitzler, J. C., Huang, S., Morishita, T. *Infect. Immun.* (2000), **68,** 5679–5689). The published secondary structure prediction and our alignment of these additional sequences confirmed our alignment of the MOMP with eighteen-stranded porins.

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