Topological investigations of the FomA porin from Fusobacterium nucleatum and identification of the constriction loop L6

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Porin FomA in the outer membrane of Fusobacterium nucleatum is a trimeric protein, which exhibits permeability properties similar to that of the well-known enterobacterial diffusion porins. The proposed topology model of the FomA monomer depicts the β-barrel motif typical of diffusion porins, consisting of 16 antiparallel β-strands. To investigate the accuracy of the FomA model and assess the topological relationship with other porins, individual deletions of variable size in seven of the eight surface-exposed regions of the porin were genetically engineered. Deletions in the predicted loops L1 to L7 were tolerated by the FomA porins, as judged by a normal assembly in the outer membrane of Escherichia coli and a sustained pore-forming ability. Deletions in the largest proposed external region, loop L6, made the FomA porins considerably more permeable to antibiotics, indicating larger pore channels. The distinctly increased uptake rates and size exclusion limits displayed by the L6 deletion mutant porins, suggest that loop L6 folds back into the β-barrel thereby constricting the native FomA channel. Thus, the position of the channel constriction loop appears to be shifted towards the C terminus in the FomA porin, as compared to the crystal structures of five non-specific diffusion porins.

Keywords: outer membrane protein, membrane topology, deletion mutagenesis, β-lactam uptake, eyelet loop

INTRODUCTION

The outer membrane of Gram-negative bacteria functions as a complex permeability barrier, protecting the bacteria from harmful agents in hostile environments (Nikaido & Vaara, 1985). Bile salts, digestive enzymes and antibiotics are denied access to the periplasm basically due to their size or polarity. Only hydrophilic solutes with molecular masses usually less than 600 Da are transported readily across the bacterial envelope through various transport proteins called porins. Such non-specific pore proteins allow free diffusion of molecules like amino acids and sugars across the outer membrane. Another class of pore proteins is substrate-specific, accelerating the uptake of certain compounds by conducting facilitated diffusion (Nikaido, 1994). Structural analysis of general diffusion porins shows that they exist as trimers of identical subunits, of which each monomer constitutes a water-filled β-barrel (Cowan et al., 1992; Weiss & Schulz, 1992). Interestingly, porins from evolutionary distant bacteria such as Rhodobacter capsulatus and Escherichia coli show remarkably similar structural features, in spite of very low sequence similarity (Cowan, 1993). The individual channels are constricted halfway through the membrane by an exterior loop, called the eyelet loop, which folds back inside the β-barrel. The crystal structures of five different porins show that loop 3 functions as the eyelet loop and that its physical parameters, such as size and charge distribution, to a large extent determine the transport properties of the channel. The other exposed regions may be involved in stabilizing the tertiary structure (Cowan et al., 1992), and function as receptor regions to phages (Egli et al., 1993), but these loops may also influence the pore permeability.

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The GenBank accession number for the sequence reported in this paper is X72583.
The anaerobic Gram-negative bacterium *Fusobacterium nucleatum* is an opportunistic pathogen suspected to play an important role in the aetiology of periodontal diseases (Kaufman & DiRienzo, 1989; Kolenbrander & London, 1993). By highly specific intergeneric co-aggregation to other bacteria, *F. nucleatum* is proposed to bridge early to late plaque colonizers, thereby introducing several anaerobic pathogens to the dental pocket. The major outer-membrane protein of *F. nucleatum* strain T18 has been shown to exhibit adhesin activity in mediating co-aggregation with *Porphyromonas gingivalis*, a species primarily found at the onset of periodontal destruction (Kinder & Holt, 1993). The corresponding 40 kDa outer-membrane protein from three *F. nucleatum* strains, termed FomA, has been shown to exhibit the properties of a general diffusion porin (Kleivdal et al., 1995), suggesting that FomA may belong to a select group of proteins with dual adhesin–porin functions. The FomA porin exists as distinct trimers in black lipid bilayers and displays a single-channel conductivity of about 1.2 nS (Kleivdal et al., 1995). The porin is weakly ion selective and the monomers close individually as a response to membrane potential shifts at ±80 mV.

The deduced amino acid sequence from four different fomA genes showed no sequence similarity to other proteins (Bolstad et al., 1994, 1995). However, the topology model of FomA (Bolstad et al., 1994), based on structure-prediction principles derived from *E. coli* outer-membrane proteins, indicates that FomA shares several structural features with the porins of other Gram-negative bacteria (Cowan et al., 1992; Weiss & Schulz, 1992). The FomA monomer is proposed to fold as a 16-stranded antiparallel β-barrel with eight loops exposed to the exterior (Bolstad et al., 1994, 1995). Most of these regions correspond to the most hydrophilic and variable regions among the fusobacterial porins, and we have recently demonstrated the surface exposure of six of the predicted loops by insertion mutagenesis (Puntervoll et al., 2000). Furthermore, a recent point mutation analysis identified two adjacent arginine residues, Arg⁸⁰ and Arg⁹², with properties consistent with a location at key positions in the positive cluster at the pore constriction of FomA (Kleivdal et al., 1999). The results were analogous to observations made with porin OmpF of *E. coli* with point mutations in positions Arg⁴² and Arg⁸² situated on the barrel wall inside this pore channel (Karshikoff et al., 1994; Saint et al., 1996). Juxtaposed to these key arginines, several acidic residues protrude into the channel lumen from the eyelet loop, thereby creating the characteristic transverse electrostatic field across the pore constriction of OmpF. The existence of a pore constriction in FomA similar to that of the classical porins would also imply the existence of a negative cluster in the channel lumen, possibly situated on a putative constriction loop. In this study, we have employed deletion mutagenesis to investigate both the validity of our putative topology model of FomA, and to what extent the topology of this porin is in agreement with the topology of the classical porins. Individual deletions of various extents were constructed in seven of the eight proposed surface-exposed loops of the FomA porin. One would expect that the deletions comprising complete or large fragments of the transmembrane segments would not be tolerated (Agterberg et al., 1989; Bosch et al., 1986). Founded on these principles, several topological models of porins have been assessed by deletion analysis (Huang et al., 199; Klebba et al., 1994; Srikumar et al., 1997). In addition to the topological information, this approach was expected to provide information on the individual loops: whether they are responsible or irrelevant for the structural and functional integrity of the FomA protein. Deletions in the eyelet loop of *E. coli* porins have been shown to produce channels with increased size-exclusion limits, able to facilitate uptake of large sugar compounds and antibiotics (Benson & Decloux, 1985; Roque & McGarhoury, 1990; Saint et al., 1996). Several deletion mutants of the FomA protein were characterized and the functional consequences on porin activity were studied in vivo. The results provide new information on the predicted topology of the FomA porin and contribute to a further understanding of porin diversity.

**METHODS**

**Bacterial strains and growth conditions.** The recombinant FomA protein was expressed in *E. coli* K-12 strain CE1224, which is devoid of all general diffusion porins due to mutations in the pboE and ompR genes (Tommassen et al., 1983). The CE1224 cells were grown in Luria–Bertani medium at 37°C overnight. To induce expression of FomA from the pboE promoter, overnight cultures of CE1224 cells grown in Luria–Bertani medium were washed once in low phosphate medium and resuspended in low phosphate medium to an OD₆₀₀ of 0.15 (Kleivdal et al., 1999; Levinthal et al., 1962). Chloramphenicol (50 µg ml⁻¹) and/or ampicillin (50 µg ml⁻¹) were used in selective media when required.

**Generation of random length deletions by Bal-31 exonuclease.** The *fomA* gene from *F. nucleatum* strain ATCC 10953 has been cloned into plasmid pHB14, downstream of the signal-sequence-encoding DNA of pboE (Jensen et al., 1996). By BamHI linker insertion mutagenesis and site-directed mutagenesis, several pHB14 derivatives were made containing unique BamHI sites at various positions in the *fomA* sequence (Puntervoll et al., 2000). With pHB14 as a template, a BamHI site after codon 60 in the *fomA* gene, corresponding to loop L2 of the protein, was created by the PCR overlap extension method using a mismatch primer (Ho et al., 1989). To create random length deletions in the *fomA* gene, 2 µg plasmid pHB14 was linearized with BamHI at unique sites. After isolating the linear DNA from agarose gels using the JetSorb gel extraction kit (Genomed), 0.5 units exonuclease Bal-31 was added in the buffer supplied by the manufacturer (Promega), and the mixture was incubated at 30°C in aliquots for 20 s, 40 s, 1 min, 2 min and 3 min. To stop the reaction 10 mM EGTA, pH 8.0 was added. The aliquots were pooled, phenol-extracted and the DNA was ethanol precipitated as described by Sambrook et al. (1989). The truncated pHB14 plasmids were religated in 20 µl ligation buffer containing 5 U T4 DNA ligase (Promega) and 1 U BamHI to ensure religation of truncated plasmids only. The ligation products were used to transform strain CE1224 as described by Sambrook et al. (1989), with selection for chloramphenicol resistant colonies.
Membrane topology of FomA

**Fig. 1.** Location of deletions within the proposed topology model of FomA from *F. nucleatum* ATCC 10953 (Bolstad et al., 1994). The view is from the unrolled β-barrel with the predicted transmembrane segments presented as tilted boxes and loop regions as circles. The shaded boxes indicate residues facing the lipid environment, whereas the white boxes represent residues pointing into the channel lumen. The eight proposed surface-exposed loops are labelled L1 to L8 from the N terminus. Eleven deletions were constructed by deletion mutagenesis of *fomA*. The deleted amino acid residues are presented as unfilled letters and the positions where the individual deletions start and end are numbered (see Table 1). The numbering of aa 1–135 corresponds to the sequence published by Bolstad et al. (1994). Val136 of the published sequence has since been shown not to be present (our unpublished results), and the revised sequence data have been submitted to the GenBank database under accession number X72583.

**Generation of specific deletions.** The deletion L3 Δ98–106::RG was created by replacing the 315 bp *PstI*–*BamHI* fragment from pHK105 with the 297 bp *PstI*–*BamHI* fragment from pMB97 (Puntervoll et al., 2000). Deletion L3 Δ98–116::RSIRV was constructed by exchanging the 297 bp *PstI*–*BamHI* fragment from pMB97 and the respective 350 bp *PstI*–*BamHI* fragment from pMB116 (Puntervoll et al., 2000). To retain the reading frame of the *fomA* gene, both *BamHI* ends were made blunt by the Klenow fragment of *E. coli* DNA polymerase I (Promega) prior to ligation. Deletion L4 Δ139–145::TR was created by replacing the 430 bp *PstI*–*BamHI* fragment of pHK105 with the 412 bp *PstI*–*BamHI* fragment of pMB138 (Puntervoll et al., 2000). Prior to ligation, the *BamHI* ends were made blunt by using mung bean nuclease (Promega) to retain the reading frame. All the plasmids with truncations in the *fomA* gene were used to transform *E. coli* strain CE1224 as described above.

**Identification of rFomA deletion mutants.** Transformed cells with truncated plasmids were induced to express the rFomA proteins. Cells producing proteins with in-frame deletions in the *fomA* gene were identified by Western immunoblot analysis using anti-FomA antiserum. Plasmids from these cells were isolated and sequenced to confirm deletions in the *fomA* region. For this purpose, the plasmids were purified using the Wizard plasmid kit (Promega) and sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 377 DNA sequencer (Perkin Elmer Applied Biosystems).

**SDS-PAGE and Western immunoblotting.** Crude cell envelope preparations for SDS-PAGE analysis were obtained from 10 ml overnight cultures as described by Merck et al. (1997). Electrophoresis was carried out by SDS-PAGE in discontinuous 10% polyacrylamide gels as described by Lugtenberg et al. (1975). Before electrophoresis, the samples were incubated for 5 min in sample buffer either at room temperature or 95 °C. Proteins were stained with Coomassie brilliant blue R250 (Sigma). For quantification of FomA, protein bands were stained with the fluorescent dye SYPRO Orange according to the instructions of the manufacturer (Molecular Probes). The protein bands were visualized and analysed on a Fluorescent Image Analyser FLA-2000 (Fujifilm). Western immunoblot analysis was performed as previously described (Kleivdal et al., 1995). Anti-FomA antiserum φ239 (Bakken & Jensen, 1986) was used as the primary antibody and the blots were developed using horseradish peroxidase conjugated goat anti-rabbit antibodies (Bio-Rad).

**Functional characterization of the mutant rFomA porins by in vivo uptake of β-lactams.** The rates of permeation of the β-lactam antibiotics cephaloridine (415 Da) and cefsulodin (554 Da) through the outer membrane of intact cells were determined by the β-lactamase assay, a method originally described by Zimmermann & Rosselet (1977). In this assay,


Table 1. Mutated rFomA proteins used in this study and their permeability properties as measured by in vivo assays

<table>
<thead>
<tr>
<th>rFomA porin*</th>
<th>Rate of antibiotic uptake (%)†</th>
<th>Antibiotic growth inhibition zone (mm)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass</td>
<td>Cephaloridine§</td>
<td>Cefsulodin‖</td>
</tr>
<tr>
<td>Wild-type</td>
<td>415</td>
<td>550</td>
</tr>
<tr>
<td>L1 A8–23::RS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L2 A57–62</td>
<td>135 ± 12</td>
<td>149 ± 12</td>
</tr>
<tr>
<td>L3 A98–106::RG</td>
<td>176 ± 13</td>
<td>57 ± 6</td>
</tr>
<tr>
<td>L3 A98–116::RGISR</td>
<td>114 ± 3</td>
<td>104 ± 3</td>
</tr>
<tr>
<td>L4 A139–145::TR</td>
<td>112 ± 4</td>
<td>50 ± 13</td>
</tr>
<tr>
<td>L5 A177–192::ARKGKVHRS</td>
<td>140 ± 12</td>
<td>75 ± 8</td>
</tr>
<tr>
<td>L6 A224–236::VR</td>
<td>192 ± 23</td>
<td>182 ± 21</td>
</tr>
<tr>
<td>L6 A237–238::R</td>
<td>249 ± 6</td>
<td>283 ± 42</td>
</tr>
<tr>
<td>L6 A235–247</td>
<td>360 ± 21</td>
<td>252 ± 13</td>
</tr>
<tr>
<td>L6 A237–257</td>
<td>211 ± 13</td>
<td>241 ± 9</td>
</tr>
<tr>
<td>L7 A285–294</td>
<td>99 ± 6</td>
<td>105 ± 5</td>
</tr>
</tbody>
</table>

*Deletions are indicated by ∆ and the actual residues deleted. Insertions, due to remnants from BamHI linkers carried by the original plasmids, are indicated by double colons followed by the amino acid sequence replacing the first deleted amino acid. Position 1 is the N-terminal amino acid of the mature rFomA amino acid sequence (Fig. 1). The plasmids used to make deletions were previously described by Puntervoll et al. (2000), except the plasmid used to make the deletion in loop L2, which is described in Methods.

†Uptake of β-lactam antibiotics by E. coli CE1224 cells expressing FomA proteins. The rate of uptake was calculated as $\Delta A_{622}$ min$^{-1}$ (fluorescence unit excited from the FomA band)$^1$ on a SYPRO Orange stained gel as described. The value obtained was related to the wild-type value, and the change in uptake rate was expressed as percentage change.

‡Sensitivity to antibiotics was determined by the diameter of the growth inhibition zone, including the 6 mm filter disc containing antibiotics. The control discs, as well as the discs containing bacitracin, did not result in any growth inhibition. Results are the mean value of four individual experiments. Abbreviations: Tet, tetracycline; Neo, neomycin; Rif, rifampicin; Van, vancomycin.

§Charged molecule with a net neutral charge.
‖Charged molecule with a net negative charge.

the diffusion of externally added β-lactam antibiotics through the outer membrane is the rate-limiting step in their degradation by the periplasmic β-lactamase enzyme. The various truncated rFomA porins were expressed in E. coli strain CE1224 carrying plasmid pBR322 to ensure a high expression of β-lactamase. The assay was performed as described by Puntervoll et al. (2000). In short, the bacterial cells expressing the rFomA of interest were washed and diluted to a specific concentration the β-lactam antibiotic. A cell density of OD$_{600}$ 0.01 was used for obtaining uptake rates with cephaloridine, whereas a cell density of OD$_{600}$ 0.1 was used with cefsulodin. Final concentrations of the β-lactams were 0.8 mM and all reactions were performed at 22 °C. Five reactions were performed in parallel and stopped after 0, 5, 10, 15 and 20 min by adding an equal volume of a starch/iodeine reagent (1 M acetic acid, 0.2 M NaWO$_4$, 0.2% starch, 0.08 mM I$_2$, 3.2 mM KI). The degraded β-lactams will react with the starch/iodeine reagent and lead to a decrease in A$_{622}$, which was measured exactly 20 min after the reaction was stopped. In all uptake experiments, control samples of the cell suspensions were subjected to SDS-PAGE and stained with the fluorescent dye SYPRO Orange. The amount of the FomA protein was determined by quantifying the fluorescence from the respective protein band by using a Fluorescent Image Analyser FLA-2000 (Fujifilm). The uptake rates of each FomA mutant were expressed as $\Delta A_{622}$ min$^{-1}$ (fluorescence unit of the FomA protein bands)$^1$ and compared to that of the wild-type rFomA.

Antibiotic sensitivity assay. Sensitivity to antibiotics was examined by measuring the zone of growth inhibition around antibiotic-containing discs (Benson & Decloux, 1985). Cultures with E. coli CE1224 expressing the mutant rFomA proteins were grown to the mid-exponential phase in low phosphate medium. Low phosphate medium plates solidified with 2% agar were overlaid with 3 ml of the same medium containing 0.6% agarose and 0.2 ml mid-log culture. Whatman no. 1 paper discs (6 mm) were placed on the lawn of cells and 5 μl of an antibiotic solution was placed on top of the disc. The plates were incubated at 30 °C overnight and the zone of growth inhibition around the disc was measured. The antibiotics (molecular mass, amount per disc given in μg or International units) were tetracycline (444 Da, 5 μg), neomycin (614 Da, 5 μg), rifampicin (823 Da, 10 μg), bacitracin (1421 Da, 20 IU) and vancomycin (1485 Da, 10 μg). Control discs contained water and 0.1% SDS.

RESULTS

To test the validity of the predicted surface-exposed loops of FomA, deletions were introduced in seven of these regions (Fig. 1) using two different strategies. Site-specific deletion mutagenesis was performed to delete...
segments of the putative loops L3 and L4, by combining fomA sequences carrying unique BamHI sites at positions 97, 105, 116, 138 and 143 (Puntervoll et al., 2000), whereas deletions in loops L1, L2, L5, L6 and L7 were made randomly using nuclease Bal-31 (Table 1). In the latter case, plasmids, linearized at unique BamHI sites (Puntervoll et al., 2000), were subjected to nuclease activity, religated and used to transform the porin-deficient E. coli strain CE1224. Successful truncations of the fomA gene implicated an in-frame religation of the digested plasmid, and that the subsequent structural alterations did not impede the biogenesis of the protein. Colonies expressing such truncated rFomA porins were identified by using FomA-specific antisera on Western blots. The permisive deletions in the fomA regions were characterized by sequencing and the mutant proteins were functionally investigated. Some of the protein products carried small residual inserts originating from the BamHI linkers (RGSIRV) (Puntervoll et al., 2000). The size of the deletions varied from 1 to 20 aa, while mutants L3 Δ98–116 and L5 Δ177–192 also carried inserts of six and nine residues from the BamHI linkers respectively (Table 1).

Expression and folding of the FomA deletion mutants

All the mutant FomA porins identified were localized in the cell envelope fraction of the transformed E. coli cells, indicating a correct incorporation of the truncated proteins into the outer membrane (Fig. 2). In general, the higher electrophoretic mobilities of the denatured mutant proteins reflected the size of their deletions (Fig. 2).

An exception was deletion L6 Δ237–257, which did not significantly alter the mobility of the denatured monomer. The expression level of most of the mutant porins was comparable to that of the wild-type as seen when equal amounts of cell envelopes were analysed by SDS-PAGE. However, the cells expressing FomA porins with deletions in loop L7 and some of the deletions in loops L3 and L6 displayed slightly reduced levels of expression (Fig. 2), suggesting that these truncations may have affected the stability of the protein scaffold.

As previously reported, FomA porins from different F. nucleatum strains display a variable degree of trimer stability (Kleivdal et al., 1995). Unheated samples of the wild-type rFomA, cloned from the fusobacterial strain ATCC 10953, are mainly observed as folded monomers, which migrate faster on SDS-PAGE than the denatured rFomA (Bakken & Jensen, 1986; Jensen et al., 1996). However, trimers have been detected in black lipid bilayers and by chemical cross-linking of FomA in the outer membrane of this strain (Kleivdal et al., 1995). In this study, all the deletion mutants displayed heat modifiability, comparable to the wild-type rFomA, as judged by the migration of unheated and heated protein samples (Fig. 3). The mutant proteins displayed a weak trimer band at about 70 kDa in addition to the band of folded monomers. In the case of FomA mutants with deletions in loop L1 and L5, the electrophoretic mobility pattern of the unheated samples differed substantially from that of the wild-type protein (Fig. 3). Both the folded and denatured monomers of L1 Δ8–23::RS migrated considerably faster than those of the wild-type. Also the trimers of this mutant appeared to migrate faster than the wild-type trimers, suggesting that whatever caused the mobility effect seen with the folded monomer...
monomers also applies to the trimers. The mutant porin L5 A177–192:: ARKGVHRS apparently featured a higher portion of stable trimers than the wild-type rFomA (Fig. 3), suggesting a trimer-stabilizing effect. However, since the amount of folded monomers did not decrease concomitantly, the higher proportion of trimers may merely reflect an increased affinity between the trimers and the anti-FomA antiserum.

**Functional studies on uptake of antibiotics**

To investigate the functional effects of the deletions, the permeability properties of the truncated rFomA proteins were assessed by studying the uptake of β-lactam antibiotics in vivo. The mutant rFomA porins were expressed by the porin-deficient *E. coli* strain CE1224 and the uptake of β-lactam antibiotics was monitored colorimetrically as described in Methods. The uptake rates of two differently charged β-lactams were measured and normalized to those of the wild-type (Table 1). All the truncated proteins were still functional as porins. This strongly supports the notion that the deletions are located in external loops, leaving the general conformation of the β-barrel intact. On the other hand, most of the deletions produced a measurable effect on the rFomA permeability. The exception was proteins with truncations in loop L7, which displayed only slight permeability differences compared to the wild-type porin (Table 1).

The only mutation that caused decreased uptake rates of both β-lactams was the deletion in L1. This deletion affected a region that is highly conserved among the fusobacterial FomA porins and may therefore be of structural or functional importance (Bolstad et al., 1994). In either way, directly or indirectly, the deletion seemed to disturb the conformation, which again led to an altered electrophoretic mobility of the L1 mutant. A distinct group of deletion mutants produced porins with an increased permeability to the zwitterionic cephaloridine, but a decreased permeability to the anionic cefsulodin. This charge selectivity effect was observed with deletions L3 Δ98–106::RG, L4 Δ139–145::TR and L5 Δ177–192:: ARKGVHRS, located in the middle region of the β-barrel. Mutant L3 Δ98–116:: RGSIRV porins behaved quite similarly to the wild-type, even though part of the putative transmembrane segment β6 was implicated.

The most profound effects were observed with the four rFomA mutants carrying deletions in the proposed loop L6, which demonstrated drastically increased uptake rates of both antibiotics. The deletions were located at different parts of the loop, altogether covering the entire L6 region. One of these deletion mutants (L6 Δ235–247), carrying a 13 aa deletion in the middle of the loop, produced the highest uptake rate of cephaloridine and a considerably increased uptake rate of cefsulodin (Table 1). Another mutation substituting two lysines by one arginine at the tip of the loop (L6 Δ237–238:: R) resulted in significantly elevated uptake rates compared to the wild-type rFomA. Such severe permeability effects from a net deletion of one amino acid were quite unexpected. These distinct permeability effects strongly suggest an important functional role of loop L6. The deletion in loop L2 also produced increased uptake rates in general, but the effects were not as pronounced as the L6 deletions.

The permeability properties of the deletion mutants were further investigated by the antibiotic sensitivity disc assay. CE1224 cells expressing the mutant rFomA proteins were tested for their sensitivity to several antibiotics of different sizes (Table 1). In general, only minor variations in sensitivity towards the antibiotics tetracycline, neomycin and rifampicin were observed when comparing the various mutant rFomA proteins to the wild-type FomA. However, cells expressing the mutant protein L6 Δ235–247 were sensitive to the large antibiotic vancomycin (1485 Da), indicating that the exclusion limit of the FomA porin was affected by the deletion. None of the other deletions resulted in such an effect. In contrast to the uptake rates from the β-lactamase assay, the results from the antibiotic sensitivity disc assay were not corrected for possible variations in the rFomA expression level.

**DISCUSSION**

The basis for our topological investigation is that deletions in porins are generally found to be non-disruptive if they occur in the surface loops, which probably have more freedom to change without interrupting the biogenesis and perturbing the β-barrel conformation (Agterberg et al., 1989; Jeanteur et al., 1991). In contrast to the loop regions, the importance of intact membrane-spanning segments for the structural and functional integrity of the PhoE porin in *E. coli* has been demonstrated (Bosch et al., 1988, 1989). In the present study, deletions of variable size were constructed in seven of the eight regions proposed to constitute surface-exposed loops according to the predicted FomA model (Bolstad et al., 1994). The functional characterization of the permissive deletion mutants of FomA enabled us to assess the general accuracy of this topology model. The correct localization in the outer membrane together with sustained functional properties confirmed that the general β-barrel conformation of all the truncated rFomA proteins was maintained, probably because the deletions were confined to the interconnecting loops (Fig. 1). These results strongly suggest that the location of the first seven proposed loop regions in FomA are accurately predicted, although this approach does not permit a precise prediction of the exact amino acids at which β-strands and loop regions start and finish.

Besides the topological information obtained from the location of permissive deletions in FomA, the deletion analysis enabled us to investigate the importance of each surface-exposed region with respect to pore function. The functional effects of deletions were correlated to the proposed topology model of FomA, in accordance with the structure–function relationship established for porins with known structure (Agterberg et al., 1989;
Benson & Decloux, 1985; Lou et al., 1996; Saint et al., 1996). The strongly conserved nature of loop L1 among the FomA proteins suggests an important structural or functional role (Bolstad et al., 1994, 1995). Indeed, deletion of L1 affected the structure, giving rise to an altered electrophoretic mobility of the folded monomer and trimer, and had functional effects, observed as a decreased β-lactam permeability by the porin. In contrast, we recently showed that the insertion of an epitope into loop L1 did not cause any severe functional effect, and that the inserted epitope was not surface exposed (Puntervoll et al., 2000), despite the fact that the postulated loop L1 is accessible to trypsin proteolysis on whole cells (Bakken & Jensen, 1986). Further work is clearly needed to elucidate the functional and structural role of loop L1.

Both deletions made in the third loop region of FomA were tolerated, although mutant L3 Δ98–116::RG SIRV showed a reduced level of expression. This can be explained by the partial substitution of segment β6 with the BamHI linker residues. Although the substitution maintained the amphipathic nature of this segment, the region was probably perturbed sufficiently to partially impair the biogenesis. The loop L5 deletion also substituted a transmembrane segment (β9) by linker residues, but in this case the substitution did not preserve the amphipathic motif. Despite these alterations, the biogenesis of the mutant protein seemed similar to the wild-type porin indicating that the substitution was either tolerable to this transmembrane region or that the prediction of this region may need to be modified.

The deletions in loops L4 and L5, and deletion L3 Δ98–106::RG all produced pores more permeable to cephaloridine, but less permeable to cefsulodin compared to the wild-type porin. These loops may be oriented towards the central axis of the monomeric channel, thereby creating a wider pore entrance upon deletion. This could make the truncated pores more permeable to the neutral cephaloridine. However, the transport of the anionic cefsulodin does not depend on pore dimensions alone, but also on the charge distribution within the pore. The deletions in loops L4 and L5 and deletion L3 Δ98–106::RG may uncover the excessive negative charges, situated closer to the pore constriction, which contribute to the cation selectivity of FomA (Kleivdal et al., 1995, 1999). Hence, the negatively charged cefsulodin would experience a more restrictive pre-filtering by charge, resulting in a decreased permeability to this compound. Analogously, the deletion in loop L2 may cause a wider pore inlet, but the concurrent removal of four acidic residues in L2 may alter the charge pre-screening, causing an increased uptake of cefsulodin. As observed with the classical porins, the loss of exposed negative charges at the pore mouth may influence the ion selectivity (Cowan et al., 1992; Karshikoff et al., 1994). Interestingly, despite having a larger deletion than the L3 Δ98–106::RG porin, the L3 Δ98–116::RG SIRV porin does not seem to differ functionally from the wild-type FomA porin. A priori, one would expect a larger deletion to have a more pronounced functional effect than a smaller one, but differences in net charges may explain this apparent inconsistency. Loop L3 of the L3 Δ98–116::RG SIRV porin has a net charge of +2 as opposed to the neutral third loop of the L3 Δ98–106::RG porin. This surplus of positively charged amino acids on the smaller loop may balance the exposure of negatively charged residues situated closer to the pore restriction, and thus cancel the above discussed effects on ion-selectivity.

The four permissible deletions of L6, altogether covering the region from position 224 to 257, indicate that this region, found in all the four FomA sequences, is a long continuous loop region. The sixth loop is thus the most prominent loop in FomA, a feature also found to be characteristic for the eyelet loops displayed by the crystallized porins (Cowan et al., 1992; Hirsch et al., 1997; Kreusch et al., 1994; Weiss & Schulz, 1992). Furthermore, all the deletions introduced in loop L6 resulted in significantly increased uptake rates of β-lactams, strongly indicating an increased cross section of the FomA channel. This supposition was confirmed in the antibiotic sensitivity assay, where cells expressing rFomA L6 Δ235–247 became sensitive to vancomycin (1485 Da). As this antibiotic is larger than the exclusion limit of the wild-type rFomA channels, the deletion of loop L6 must have increased the pore diameter. This is analogous to recent results obtained with PhoE, which becomes permeable to large antibiotics when fragments from the eyelet loop are deleted (Van Gelder et al., 1997), and to observations with OmpF porins, where deletions in the eyelet loop region greatly increased the diameter at the pore constriction (Benson et al., 1988; Saint et al., 1996). The results strongly suggest that loop L6 of FomA constricts the channel and thereby fulfills the role as an eyelet loop as was previously proposed by Bolstad et al. (1994). The disproportionate permeability effect observed with mutant L6 Δ237–238::R, i.e. the substitution of two lysines with one arginine, indicates a delicate positioning of loop L6. These lysines are conserved in all FomA proteins (Bolstad et al., 1994, 1995), also suggesting an important structural or functional role.

The slight reduction of expression levels of deletion mutants L6 Δ237–257 and L6 Δ235–247 indicate that L6 may be sensitive to perturbations. This coincides with our recent study showing that an insertion of a 16 aa epitope in loop L6 at position 236 resulted in significantly reduced expression levels, while the same epitope insertion in position 245 totally impeded the FomA expression (Puntervoll et al., 2000). These effects may be explained by the general importance of the constriction loop on the folding and stability of the channel, as demonstrated by mutagenesis studies on several porins (Saint et al., 1996; Schmid et al., 1998; Van Gelder et al., 1997). From these reports, the constriction loop does not seem to be essential for maintaining the β-barrel structure, but mutant porins may become labile and poorly expressed.

The proposed eyelet loop function of loop L6 is also in accordance with recent results from point mutations...
made in FomA (Kleivdal et al., 1999). The mutagenesis of two arginines (Arg\(^{90}\) and Arg\(^{92}\)), proposed to constitute part of the positive cluster at the FomA pore constriction, caused permeability effects analogous to the key arginines in the OmpF porin (Saint et al., 1996). If loop L6 does fold back into the channel, aligned towards the C-terminus, conserved acidic residues at this loop may be juxtaposed to arginines 90 and 92 in creating a transverse electric field across the pore constriction.

In conclusion, the FomA topology model was challenged by deletion analysis and strengthened by the characterization of permissive deletions. The apparent near-native folding of the truncated FomA porins strongly suggests that the deletions presented here are situated in loop regions. Based on the distinctly increased size exclusion limit observed with L6\(\Delta\) mutants, we propose loop L6 to be the eyelet loop of the FomA porin, in contrast to the eyelet function maintained by loop L3 in other bacterial non-specific porins. In addition to the eyelet function of FomA being shifted towards the C-terminus compared to the classical porins, other features such as key arginines at the constriction site also appear to be shifted towards the C-terminus. Such a divergence from the molecular organization of the classical non-specific porins may not be unexpected, due to the lack of sequence similarity. Although some of the porins with resolved atomic structure display very low sequence similarity to one another, the bacteria from which they originated all belong to the proteobacteria phylum. In contrast, phylogenetic analysis based on small subunit rRNA sequences, places *Fusobacterium* in a separate phylogenetic group (Olsen et al., 1994). The FomA porin may therefore constitute a new subclass of non-specific diffusion porins.

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**REFERENCES**


Membrane topology of FomA


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