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Linker-insertion mutagenesis of *Pseudomonas aeruginosa* outer membrane protein OprF

Rebecca S. Y. Wong, Helen Jost and Robert E. W. Hancock*

Department of Microbiology, University of British Columbia, 300–6174 University Boulevard, Vancouver, British Columbia V6T 1Z3, Canada.

Summary

The *oprF* gene, expressing *Pseudomonas aeruginosa* major outer membrane protein OprF, was subjected to semi-random linker mutagenesis by insertion of a 1.3 kb *Hin*cII kanamycin-resistance fragment from plasmid pUC4KAPA into multiple blunt-ended restriction sites in the *oprF* gene. The kanamycin-resistance gene was then removed by *Pst*I digestion, which left a 12 nucleotide pair linker residue. Nine unique clones were identified that contained such linkers at different locations within the *oprF* gene and were permissive for the production of full-length OprF variants. In addition, one permissive site-directed insertion, one non-permissive insertion and one carboxy-terminal insertion leading to proteolytic truncation were also identified. These mutants were characterized by DNA sequencing and reactivity of the OprF variants with a bank of 10 OprF-specific monoclonal antibodies. Permissive clones produced OprF variants that were shown to be reactive with the majority of these monoclonal antibodies, except where the insertion was suspected of interrupting the epitope for the specific monoclonal antibody. In addition, these variants were shown to be 2-mercaptoethanol modifiable, to be resistant to trypsin cleavage in intact cells and partly cleaved to a high-molecular-weight core fragment in outer membranes and, where studied, to be accessible to indirect immunofluorescence labelling in intact cells by monoclonal antibodies specific for surface epitopes. Based on these data, a revised structural model for OprF is proposed.

Introduction

The outer membranes of Gram-negative bacteria are asymmetric bilayers consisting of lipids or phospholipids in the inner monolayer and lipopolysaccharide (LPS) in the outer monolayer with a restricted number of proteins inserted into either or both monolayers. The outer membrane represents a primary barrier between the cell and the environment. Therefore, it plays an important role in determining the entry of substances into the cell. Certain proteins in the outer membrane, known as porins, form aqueous channels which allow the passage of hydrophilic molecules. The major outer membrane protein of *Pseudomonas aeruginosa*, OprF, is a porin (Benz and Hancock, 1981; Bellido *et al.*, 1992). In addition, OprF contributes to the structural integrity of the outer membrane and the cell in maintaining cell morphology and enabling cell growth in low osmolarity media (Woodruff and Hancock, 1989).

Based on data obtained from circular dichroism studies and the protein structure-prediction method of Paul and Rosenbusch (1985), Siehnel *et al.* (1990) described a working model for the topological organization of OprF in the outer membrane. This model has been tested in part by Tn*phoA* mutagenesis and deletion analysis of the *oprF* gene, which permitted the partial delineation of the epitopes recognized by 10 different OprF-specific monoclonal antibodies (Finnen *et al.*, 1992), nine of which recognize surface epitopes (Martin, 1992). The epitope locations were further defined by utilizing chemical and proteolytic digestion products of purified OprF (Martin, 1992). Both of these studies have generated a significant amount of data that can be used in defining the membrane topology of OprF. These studies provided evidence for the presence, on the surface of the bacterium, of several portions of the carboxy-terminus of OprF, despite the fact that the homologous region of the related (Duchene *et al.*, 1988; Woodruff and Hancock, 1989) protein OmpA from *Escherichia coli* had been assigned to the periplasm (Freudl *et al.*, 1986). Nevertheless, these approaches had limitations in that the epitopes could only be narrowed down to regions of between 26 and 65 amino acids.

Linker-insertion mutagenesis, either random or restriction site-directed, has been employed to study the topology of several *E. coli* outer membrane proteins including the maltoporin LamB (Boulain *et al.*, 1986), the phosphate-starvation-inducible porin PhoE (Bosch and Tommassen, 1987) and the major peptidoglycan-associated protein OmpA (Freudl *et al.*, 1986). Linker insertion introduces a stretch of several amino acid residues in the

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amino acid sequence of these membrane proteins, while leaving the rest of the protein intact. As a result, it represents a more subtle modification of the protein in comparison to the approaches previously utilized for OprF. A recent crystal structure of PhoE (Cowan *et al.*, 1992) has demonstrated that this protein contains a rigid β-barrel structure of 16 transmembrane β-sheet strands of 9–16 amino acids, separated by loop regions. Generally speaking, these loop regions of outer membrane proteins are more likely to accommodate extra amino acids without gross perturbation of the protein structure. Indeed all of the known PhoE insertion sites (Cowan *et al.*, 1992) occur within these loops. If such insertions affect the binding of monoclonal antibodies that recognize cell surface-exposed epitopes, it is thus possible to determine the surface exposure of the insertion sites.

In this paper, we present our work on semi-random linker-insertion mutagenesis of the cloned *oprF* gene. The resultant mutants have permitted the generation of a refined membrane topology model for OprF and definition of specific sites involved in reactivity of certain monoclonal antibodies.

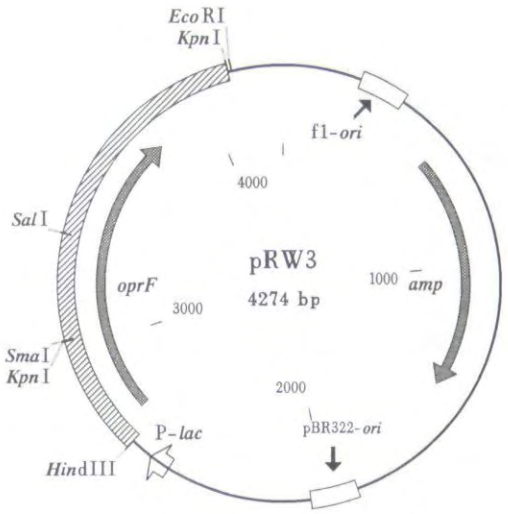

Fig. 1. Restriction map of plasmid pRW3. The position and direction of transcription of *oprF* and the ampicillin-resistance marker (Amp) are indicated. Abbreviations: f1-*ori*, f1 origin of replication; pBR322-*ori*, pBR322 origin of replication; P-*lac*, *lac* promoter; bp, base pairs.

Results

Construction of pRW3

It was not possible to subclone the native *oprF* gene into high-copy-number plasmids, probably because of the efficient expression of OprF from its own promoter in *E. coli* leading to overexpression lethality (Woodruff *et al.*, 1986). Therefore the putative –10 site of the *oprF* gene was mutated by adding a G:C nucleotide pair between nucleotides –9 and –10 to create a *Hin*dIII site. This weakened the *oprF* promoter, permitting the *oprF* gene to be subcloned into high-copy-number plasmids. The final constructed plasmid, pRW3 (Fig. 1), contained no *Pst*I sites, permitting subsequent introduction of a *Pst*I site during linker-insertion mutagenesis, as well as a unique *Sal*I site within the *oprF* gene. Furthermore, the removal of most of the *oprF* gene-flanking DNA sequences maximized the efficiency of linker-insertion mutagenesis. *E. coli* strains containing pRW3 expressed OprF, which was detectable by all 10 OprF-specific monoclonal antibodies on Western immunoblots. Induction of the *lac* promoter with IPTG (1 mM) for 4 h before harvesting only increased the expression by approximately twofold, despite the fact that the *lac* promoter was oriented in the same direction as the *oprF* gene.

Linker mutagenesis

The plasmid pRW3 was linearized separately by partial digestion with each of the four restriction enzymes as described in the *Experimental procedures*. There were a total of 74 cleavage sites in pRW3 that were recognized by the four enzymes utilized; 37 of these were within the *oprF* gene. Low enzyme concentrations and/or ethidium bromide were used to favour the production of singly cut plasmids, which were further purified by elution from preparative agarose gels. After ligation of the restriction enzyme-linearized plasmid pRW3 with the 1.3 kb kanamycin-resistance cassette, 240 of the 450 screened transformants showed an OprF-deficient and kanamycin-resistant phenotype, indicating that the insertion had occurred within the *oprF* gene. Plasmid DNAs were prepared from 100 clones and digested with *Pst*I to remove the kanamycin-resistance cassette but leaving a residue of 12 nucleotide pairs, which was between the *Hin*cII sites and *Pst*I sites flanking both sides of the cassette. After religation, 44 of the 100 kanamycin-sensitive clones had regained the ability to express OprF, as determined by colony immunoblot with monoclonal antibody MA5-8. Thus these clones represented insertion sites that were permissive for the insertion of four extra amino acids. The rest of the kanamycin-sensitive clones were unable to express OprF or reacted only weakly with the OprF-specific monoclonal antibody MA5-8 on colony immunoblots. Restriction enzyme analysis was used to map the insertion sites in each of the 100 plasmids, which categorized the sites in the 44 OprF-expressing plasmids into 10 unique groups. The remaining 56 plasmids included a number which demonstrated gene rearrangements or deletions, probably owing to multiple cleavages of pRW3 by the restriction enzymes prior to ligation with the mutagenesis cassette. Sequencing of the plasmid DNA from

eight of the kanamycin-sensitive, OprF-non-reactive mutants revealed that two of the mutants had incorporated the 12 nucleotide pair insert in a reading frame that leads to the translation of a stop codon, while five other mutants represented deletions of part of the *oprF* sequence. Only one (pRW303) of the eight clones analysed showed the incorporation of a 12 nucleotide pair insert without any genetic alteration or change of reading frame. The inability of this clone to demonstrate an OprF positive phenotype on colony or Western immunoblot suggested the 'non-permissiveness' of this insertion site.

Characterization of linker insertion mutants

The exact linker-insertion sites in at least one representative from each of the 10 groups of the OprF-expressing mutant plasmids were determined by DNA sequencing. The nucleotide positions of the linker insertions and the identities of the inserted four amino acids for 11 linker insertion mutants (including one non-permissive mutant) and one site-directed insertion mutant (pRW307) are summarized in Table 1. In addition, two other plasmids contained inserts at nucleotides 491 and 853 that were translated to premature stop codons (pRW304 and 315 respectively) and thus were not further considered here. The expression of OprF from the 12 mutated plasmids was examined by SDS–PAGE of outer membrane samples containing the mutated proteins (Fig. 2). All of the mutated proteins had mobilities that were modified by pretreatment with 2-mercaptoethanol, indicating that the inserted amino acid residues did not perturb the formation of the OprF cysteine disulphides. The apparent molecular masses of two of the mutated proteins produced by plasmids pRW301 and pRW305 were noticeably greater than that of the wild type (Table 1). However, the protein expressed by pRW305 ran with a mobility similar to the unfolded, heat-modified form of OprF as visualized on a Western blot with an OprF-specific monoclonal antibody (Fig. 3, lane 3), suggesting that the four-amino-acid linker may have influenced the susceptibility of the protein to denaturation by heating in SDS. Plasmids pRW309 (Fig. 2, lane 9) and pRW311 (data not shown) directed the expression of an intense band of apparent molecular weight 70000, probably corresponding to a protein OprF trimer (Mutharia and Hancock, 1985). After 2-mercaptoethanol treatment, a much more intense monomer band of apparent molecular weight 35000 was observed in the same samples (e.g. Fig. 2, lane 5). This suggested that insertion of the linker at amino acid positions 211 and 231 may enhance the association of SDS-stable oligomers. Two of the mutated proteins, encoded by pRW302 and 306, were produced in lesser amounts than the others, as determined by their abundance relative to the other *E. coli* proteins, indicating that insertions at these sites may lead to reduced protein production or unstable products. This was not, apparently, caused by a growth defect since the

Table 1. Insertion sites of the 11 linker insertion mutants and one site-directed insertion mutant and identities of the inserted amino acids.

Plasmids	Insertion sites (nucleotide)[a]	Insertion sites (amino acid)	Amino acids inserted	Apparent molecular mass[d] (kDa)	Apparent Molecular Mass after Trypsin Treatment[d,g] (kDa)	
					outer membranes	whole cells
pRW301	135	Gly-2	TCRS	41	ND	ND
pRW302	206	Ala-26[b]	PAGP	36	36,28	35
pRW303	256	Glu-42	DLQV	–	–	–
pRW305	521	Ala-131[b]	PAGP	40[h]	24,20,18[h]	40[h]
pRW306	534	Gly-135	TCRS	35	35,28	35
pRW307[e]	694	Val-188	DLQV	35	24,35	35
pRW308	716	Ala-196[b]	PAGP	35	35,24,28	35
pRW309	768	Arg-211	TCRS	35	28	35
pRW310	775	Asp-215	DLQV	35	28	35,28
pRW311	822	Ser-231[c]	TCRS	36	28	ND
pRW312	997	Arg-290	TCRS	35	28	28
pRW314	1059	Gly-310	TCRS	28[f]	ND	28

a. Position 1 is the transcription start site.
b. The alanine residue at the insertion site was replaced by a glycine.
c. The serine residue at the insertion site was replaced by an arginine.
d. As estimated on Western immunoblot of outer membrane samples with MA7-1. Molecular mass of unmodified OprF was 35 kDa; –, no OprF detected.
e. pRW307 was generated by inserting a *Sal*I adaptor that contained a *Pst*I site into the *Sal*I site corresponding to amino acid 188.
f. 35 kDa was observed as a minor band reactive with MA5-8.
g. Where more than one band appeared, they are listed in order of abundance, — no product; ND, not determined. pRW3-derived OprF gave a result identical to that of pRW306.
h. Tested with monoclonal antibody MA4-4 since this mutant OprF derivative was non-reactive with MA7-1.

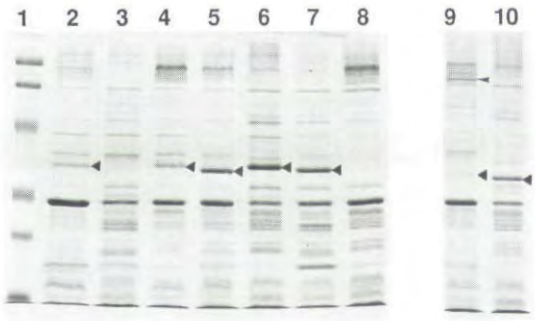

Fig. 2. SDS–PAGE of outer membrane samples of *E. coli* DH5αF' strains expressing the pRW3-derivative plasmids. Samples were incubated at 37°C for 10 min in reduction mix (2% SDS, 10% glycerol, 62.5 mM Tris pH 6.8) with (lanes 2–8) or without (lanes 9 and 10) 4% 2-mercaptoethanol before loading. Each lane contained ~16 μg protein from each sample. Plasmids present were lane 2, pRW302; lane 3, pRW305; lane 4, pRW306; lane 5, pRW309; lane 6, pRW310; lane 7, pRW3; lane 8, pTZ19R; lane 9, pRW309 and lane 10, pRW310. Lane 1 contained the molecular weight markers: phosphorylase B (106 000), bovine serum albumin (80 000), ovalbumin (49 500), carbonic anhydrase (32 500), soybean trypsin inhibitor (27 500), lysozyme (18 500). OprF monomer bands are indicated by triangles; the OprF SDS-stable trimer is indicated by an arrow head.

growth rate of *E. coli* strains carrying parental (pRW3) or any of the mutated plasmids was not different. Mutants with insertion sites in the *C*-terminal end of the proteins (e.g. those encoded by pRW312 and pRW314) produced OprF variants that were substantially but not completely degraded to smaller fragments, which included a predominant 28 kDa fragment (Table 1), confirming the results of Finnen *et al.* (1992), which indicated that the *C*-terminal regions are required for the resistance of the protein to cellular proteases.

Monoclonal antibody reactivities of mutated OprF proteins

The mutated OprF proteins of outer membrane samples were further characterized by Western immunoblot and colony immunoblot analyses, using a series of OprF-specific monoclonal antibodies (Table 2). The results demonstrated that the OprF derivatives expressed by five of the plasmids (pRW301, 302, 306, 309 and 310) were reactive with all 10 monoclonal antibodies, indicating the retention of native OprF structure. In six other mutants, specific OprF epitopes were disrupted by the insertion of the four-amino-acid linker. However, the positive reactivities of these mutated proteins with the majority of the monoclonal antibodies suggested the retention of substantial native OprF structure in these mutants. OprF expressed by pRW303 was an exception. Despite the fact that DNA sequencing demonstrated that only 12 nucleotides were inserted and no premature stop codons or changes in reading frame occurred, it did not produce any forms of OprF that could be detected by the OprF-specific monoclonal antibodies or visualized in Coomassie brilliant blue-stained gels of outer membrane samples or whole-cell lysates. Thus we assume that this represents a non-permissive insertion site (Charbit *et al.*, 1991).

Configuration of OprF in the outer membrane of E. coli

To permit conclusions to be drawn based on linker insertion mutagenesis in *E. coli,* regarding the structure of OprF, it was necessary to examine whether the structure of OprF and its mutated variants in *E. coli* reflected that of OprF in *P. aeruginosa*. It was noted that all OprF variants cofractionated with the *E. coli* outer membrane in fractionation experiments. In addition, both OprF and its variants produced in *E. coli* were 2-mercaptoethanol modifiable, suggesting that they contained the usual cysteine disulphide bonds observed with OprF in *P. aeruginosa* (Hancock and Carey, 1979). In order to probe the configurations of the OprF derivatives in the outer membrane of *E. coli*, we also conducted trypsin-accessibility assays and indirect immunofluorescence labelling.

Outer membrane porins tend to be protease resistant (Paul and Rosenbusch, 1985) by virtue of their possession of extensive β-structures with the linking surface loops tightly packed or folded in towards the porin channel (Cowan *et al.*, 1992). It was previously demonstrated that pure OprF or OprF in outer membranes was partly cleaved by trypsin to a core 28 kDa fragment, but that increasing concentrations of trypsin or increasing length of treatment time failed to cause further proteolysis

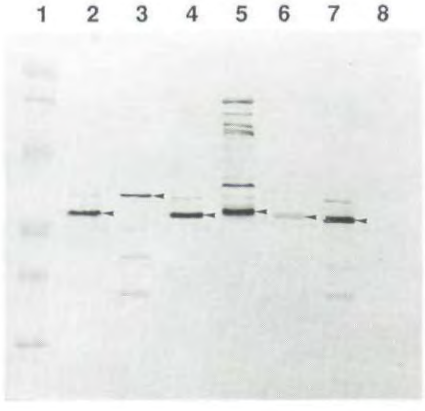

Fig. 3. Western immunoblot analysis of outer membrane samples of *E. coli* DH5αF' strains expressing the pRW3-derivative plasmids. The OprF-specific monoclonal antibody MA7-5, whose epitope is not interrupted by the insertions, was used. Samples were heated at 90°C for 10 min in reduction mix before loading. Each lane contained about 8 μg protein. Plasmids present were pRW302 (lane 2), pRW305 (lane 3), pRW306 (lane 4), pRW309 (lane 5), pRW310 (lane 6), pRW3 (lane 7), pTZ19R (lane 8). Lane 1 contained the molecular weight markers as in Fig. 2. OprF monomer bands are indicated by arrow heads.

Table 2. Summary of monoclonal antibody[a] reactivities of mutated OprF variants contained in outer membrane samples.

Plasmids	Insertion sites (amino acid position)	Monoclonal Antibody Reactivities[b]									
		7-1	7-2	7-3	7-4	7-5	7-6	7-7	7-8	4-4	5-8
pRW301	Gly-2	W	+	+	+	+	+	+	+	+	+
pRW302	Ala-26	+	+	+	+	+	+	+	+	+	+
pRW303	Glu-42	–	–	–	–	–	–	–	–	–	–
pRW305	Ala-131	–	+	+	+	+	+	+	+	+	+
pRW306	Gly-135	W	+	+	+	+	+	+	+	+	+
pRW307	Val-188	+	+	+	+	+	+	+	–	+	+
pRW308	Ala-196	+	+	+	+	+	+	+	–	–	+
pRW309	Arg-211	+	+	+	+	+	+	+	+	+	+
pRW310	Gln-215	+	+	+	+	+	+	+	+	+	+
pRW311	Ser-231	+	+	–	–	–	+	–	+	+	+
pRW312	Arg-290	+	+	–	–	–	+	–	+	+	+
pRW314	Gly-310	+	W	–	–	–	W	–	+	+	–

a. The epitopes recognized by the monoclonal antibodies are: amino acid 24 – amino acid 188 (MA7-1), amino acid 198 – amino acid 275 (MA7-2), amino acid 188 – amino acid 245 (MA7-3), amino acid 188 – amino acid 275 (MA7-4, MA7-5, MA7-7), amino acid 198 – amino acid 240 (MA7-6), amino acid 176 – amino acid 187 (MA7-8), amino acid 176 – amino acid 187 (MA4-4), amino acid 300 – amino acid 320 (MA5-8), (Martin, 1992).

b. Measured both by colony immunoblot and Western immunoblot of outer membrane samples. Symbols: +, reactivity equivalent to wild type (pRW3 plasmid-containing *E. coli*); –, no reactivity; W, weak reactivity.

(Mutharia and Hancock, 1985). Trypsin treatment of outer membranes from *E. coli* DH5αF' containing the parental plasmid pRW3 resulted in substantial retention of full-sized OprF and partial proteolysis to a 28 kDa fragment (Fig. 4, cf. lanes 8, 9, and 10). Similar results were obtained after trypsin treatment of outer membranes from cells containing plasmids pRW302 and pRW306 with *N*-terminal insertions in OprF (Fig. 4, lane 1; Table 1). Plasmids pRW309, pRW310, pRW311 and pRW312 with *C*-terminal insertions in OprF produced only the 28 kDa fragment after trypsin treatment (Fig. 4, lanes 4–7; Table 1). Plasmids pRW307 and pRW308, with insertions in the central cysteine disulphide region resulted in production of an OprF derivative that was cleaved by trypsin to a 24 kDa fragment, instead of (pRW307; Fig. 4, lane 2) or in addition to (pRW308; Fig. 4, lane 3) the 28 kDa fragment. Based on previous studies (Finnen *et al.*, 1992), such a 24 kDa fragment might be expected if cleavage occurred near amino acid 200 within the cysteine disulphide region, suggesting localized modification of OprF by this insertion which rendered the region susceptible to trypsin. Plasmid pRW305 directed expression of an OprF variant, which showed unpredictable trypsin-cleavage patterns, demonstrating a predominant 24 kDa, and minor 20 kDa and 18 kDa products (Table 1). Trypsin treatment of intact cells of *E. coli* DH5αF' containing the above plasmids did not result in proteolysis of OprF or its insertion-mutant derivatives (Table 1), with the exception of those *C*-terminal insertion mutants produced by plasmids pRW310 and pRW312.

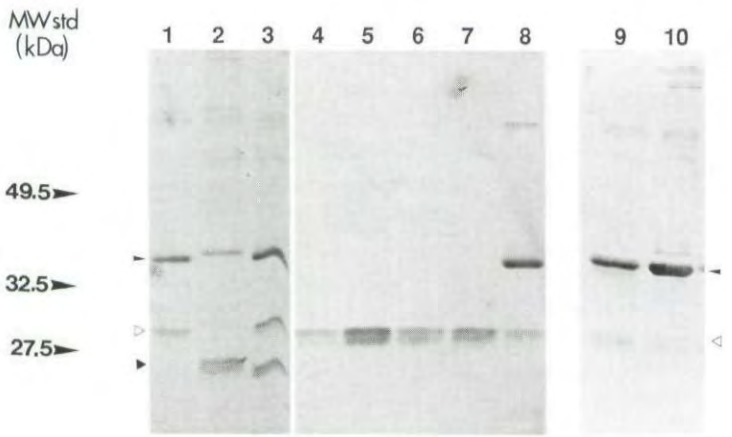

Fig. 4. Western blot analysis of trypsinized outer membrane samples containing OprF variants. Samples were treated with trypsin (0.1 mg ml^{-1}) at 37°C for 60 min, and then heated at 88°C for 10 min in reduction mix. The plasmids corresponded to the OprF variants contained in each lane are: 1, pRW302; 2, pRW307; 3, pRW308; 4, pRW309; 5, pRW310; 6, pRW311; 7, pRW312; 8, pRW3; 9, pRW3; 10, pRW3 (untreated). OprF monomer band is indicated by an arrow head. The 28 kDa and 24 kDa trypsin-resistant core fragments are indicated by open and solid triangles, respectively. Monoclonal antibody MA7-1 was used for immunodetection.

To examine if the OprF variants are surface exposed, immunofluorescence labelling was carried out using selected plasmid-containing *E. coli* strains and monoclonal antibodies against surface epitopes from the *N*-terminus (MA7-1), central region (MA7-8), and *C*-terminus (MA5-8) of OprF (Tables 2 and 3). In each case, regardless of the trypsin susceptibility of the respective OprF variants in intact cells, immunoreactivity followed precisely the pattern observed in both colony immunoblots and Western blots (Table 3). Taken together, the OprF variants are probably inserted in the outer membrane in the proper manner, as reflected by protection from trypsin and surface exposure.

Discussion

The results presented here demonstrate the construction of 10 different permissive OprF variants (including one site-directed mutant pRW307) with four extra amino acids located at sites spread throughout the OprF sequence. In addition, one partially permissive variant that was truncated by cellular proteases to a 28 kDa core in cells containing plasmid pRW314, and one non-permissive clone pRW303 were identified. The result for the plasmid pRW314-containing strain was reminiscent of the data obtained for *C*-terminal deletion variants of OprF produced by Tn*phoA* mutagenesis and subcloning (Finnen *et al.*, 1992).

To permit utilization of these data for building a revised OprF model it was first necessary to demonstrate that OprF adopted a configuration in *E. coli* identical or similar to that adopted in *P. aeruginosa*. It was previously shown that OprF in *E. coli* was both heat- and 2-mercaptoethanol-modifiable (Woodruff *et al.*, 1986; Finnen *et al.*, 1992), implying the existence of extensive β-sheet structure (Siehnel *et al.*, 1990) and the presence of intra-chain disulphide bonds (Hancock and Carey, 1979; Siehnel *et al.*, 1990; Finnen *et al.*, 1992), respectively. Furthermore, OprF purified from *E. coli* strains demonstrated similar porin function to OprF from *P. aeruginosa* (Woodruff *et al.*, 1986) and OprF had a similar shape-determining role in an appropriate *E. coli* genetic background (Woodruff and Hancock, 1989). In addition, OprF was exported to the *E. coli* outer membrane (Woodruff *et al.*, 1986) and reacted in intact cells with monoclonal antibodies specific for three surface epitopes (Finnen *et al.*, 1992) as confirmed here (Table 3). Most of the above properties were also shared by the OprF insertion mutants studied here. The demonstration of a trypsin-resistant core structure in most variants in isolated outer membranes and the general resistance of OprF to trypsin cleavage in intact cells (Table 1, Fig. 4) argued against an aberrant configuration for OprF, as did the apparently correct formation of cysteine disulphides as judged by the normal 2-mercaptoethanol modifiability of the OprF variants. In addition, most clones reacted with monoclonal antibodies MA7-3, 4, 5, 7 and 8 and MA4-4 which apparently recognize conformational epitopes (Finnen *et al.*, 1992; Martin, 1992). Furthermore, cofractionation with outer membranes and accessibility to immunofluorescence labelling in intact cells were consistent with correct surface localization of the OprF insertion variants. Taken together, the above data provide a compelling argument that the structures of OprF and its variants in *E. coli* clones are highly similar to the structure of OprF in *P. aeruginosa*, with some localized perturbations.

The 10 permissive insertion-mutant derivatives of OprF did vary somewhat when compared to unaltered OprF despite their many similarities to wild type. For example, the insertion at amino acid two of the mature protein (pRW301) directed the expression of a 41 kDa band (data not shown). Since one of the inserted amino acids was arginine, it is possible that this 41 kDa band represented OprF with the signal sequence still attached, perhaps because of defective signal peptide processing (Li *et al.*, 1988). Despite the observation that this OprF variant cofractionated with the outer membrane, results obtained by indirect immunofluorescence labelling with monoclonal antibody suggested that a derivative of this OprF variant was not surface localized. Therefore this apparent localization may have been caused by contamination of the outer membrane fraction with inclusion bodies. Plasmids pRW302 and pRW306 directed the expression of lower amounts of OprF than the other plasmids. Since the residual OprF demonstrated wild-type properties and since no 28 kDa protease cleavage fragment was observed in intact cells, we assume that these insertions influenced expression or secretion rather than stability. Plasmid pRW305 directed the expression of an OprF variant of molecular mass identical to the heat-modified, unfolded form of OprF and with a distinct trypsin-cleavage pattern. Thus we assume that this insertion influenced the stability

Table 3. Results from indirect immunofluorescence labelling of intact *E. coli* C386 cells containing various plasmids.

	Immunofluorescence with Monoclonal Antibodies[a]		
Plasmids	MA7-1[b]	MA7-8[b]	MA5-8[b]
No plasmid	–	–	–
pRW3	++	++	+
pRW302	++	++	+
pRW307	++	–	+
pRW308	+	–	+
pRW312	++	++	+

a. ++, positive labelling; +, weak labelling; –, negative labelling.
b. The epitopes recognized by these antibodies are listed in the footnote of Table 2.

of the protein to SDS, probably because of a slight change in membrane configuration. Clones containing plasmids with *C*-terminal insertions had enhanced susceptibility to trypsin cleavage in outer membranes, and to a lesser extent in intact cells. We assume that the insertions caused some localized disordering of the *C*-terminal region of OprF, perhaps preferentially exposing a trypsin-cleavage site on the periplasmic side of OprF. Plasmids pRW307 and pRW308 led to OprF variants with a different trypsin-cleavage pattern in outer membranes, possibly owing to exposure in these clones of a trypsin-accessible cleavage site in or adjacent to the cysteine disulphide loop. The insertions in pRW309 and pRW311 appeared to promote OprF trimer stability in the absence of 2-mercaptoethanol (Fig. 1, lane 9). The insertion in plasmid pRW312 led to an OprF variant that had reduced stability to cellular proteases; however 50% or more of this OprF variant remained intact and this clone was reactive with the *C*-terminal specific monoclonal antibody MA5-8 (Tables 1 and 2).

According to restriction enzyme site analysis, there were 37 sites within the *oprF* gene that were potential targets for the linker insertion mutagenesis, although we isolated insertions in only 13 of them in this study (including two of them where the inserted 12 nucleotide pairs were translated to a stop codon). This could be because insufficient clones were analysed to exhaust all the possibilities. Alternatively, it could be due to the incompatibility of the inserted amino acids with the local OprF sequences (e.g. insertion into β-strands), which might then lead to lethality of the corresponding clones or proteolytic degradation of OprF (since relatively few non-permissive insertions were further analysed in this study). It is reasonable to speculate that the nature of the amino acids in the linker might affect the result. For instance, we do not exclude the possibility that cysteine residues in the linkers translated as TCRS could disrupt OprF structure if inserted near the proposed disulphide bonds of OprF. However, the observation that the OprF variants expressed by pRW306 and pRW309 (insertion sites at amino acid 135 and 211 respectively) migrated with similar mobility on SDS–polyacrylamide gels, showed similar 2-mercaptoethanol modifiability and reacted with MA7-8 and MA4-4, which recognize epitopes sensitive to reduction of the OprF disulphide bonds, suggested that the cysteine residue present in the linkers of these variants did not participate in disulphide bonding with the endogenous cysteine residues in the protein. Nevertheless, the inserted linkers could cause minor perturbations in local conformation, even though our findings strongly suggested that the overall wild-type arrangement of OprF was still maintained. For example, the variant protein expressed by pRW309 showed a slightly different 2-mercaptoethanol modifiability compared to the wild-type protein (Figs 2 and 3, lane 5), which may be due to a change of conformation in the disulphide bond region.

Overall, the above differences in properties between wild-type and insertion-mutant derivatives are similar to those observed for LamB (Boulain *et al.*, 1986) and PhoE (Bosch and Tommassen, 1987). Interestingly, despite these differences in properties, a recent three-dimensional structure of PhoE (Cowan *et al.*, 1992) was consistent with the placement of all known PhoE insertion sites within loop regions between β-sheet strands. Therefore, we propose that the insertion sites defined here would have similar value in defining OprF topology.

Siehnel *et al.* (1990) previously presented a model for OprF based on circular dichroism data suggesting that 62% of the secondary structure of OprF was in the form of β-sheet (a value typical for outer membrane proteins (Cowan *et al.*, 1992)), the apparent existence of two disulphide bridges between the four cysteines of OprF (Hancock and Carey, 1979) and the β-turn prediction rules of Paul and Rosenbusch (1985). However, the assignment of amino acids to transmembrane β-sheets or β-turns in this earlier model was inconsistent with the data presented here since the structural model of PhoE (Cowan *et al.*, 1992) had proved the assumption that the sites of insertion of extra amino acids were the loops connecting adjacent transmembrane β-strands. Furthermore, the three solved outer membrane porin structures to date (Cowan *et al.*, 1992; Weiss *et al.*, 1991) reveal that the longest loop regions are those exposed to the surface rather than the periplasm, leading to the conclusion that insertion might occur preferentially at the surface. Therefore the primary assumption utilized in constructing our new topological model of OprF (Fig. 5) was the placement of the 13 insertion sites. It was considered that the site of amino acid number two in pRW301 was probably periplasmic since all outer membrane proteins studied to date have *N*-termini that face the periplasmic side. In addition, the insertion in pRW303 was considered non-permissive since it resulted in no detectable product. Thus the site in pRW303 was placed within the membrane. Although pRW314 resulted in production of truncated OprF, its insertion site was within a flexible protein segment adjacent to a variable sequence (see below) and thus was placed at the surface. The remaining nine sites were placed in surface loops. Six of these interrupted the binding of specific monoclonal antibodies (Table 2). In each case, these sites were within the regions of OprF to which these epitopes had been previously mapped (Finnen *et al.*, 1992; Martin, 1992). Two of the insertions interrupted binding of four monoclonal antibodies, a result consistent with data indicating that these monoclonal antibodies MA7-3, 4, 5 and 7 recognize overlapping conformational epitopes on the surface of OprF (Finnen *et al.*, 1992; Martin, 1992). In addition, MA7-8 and MA4-4 were

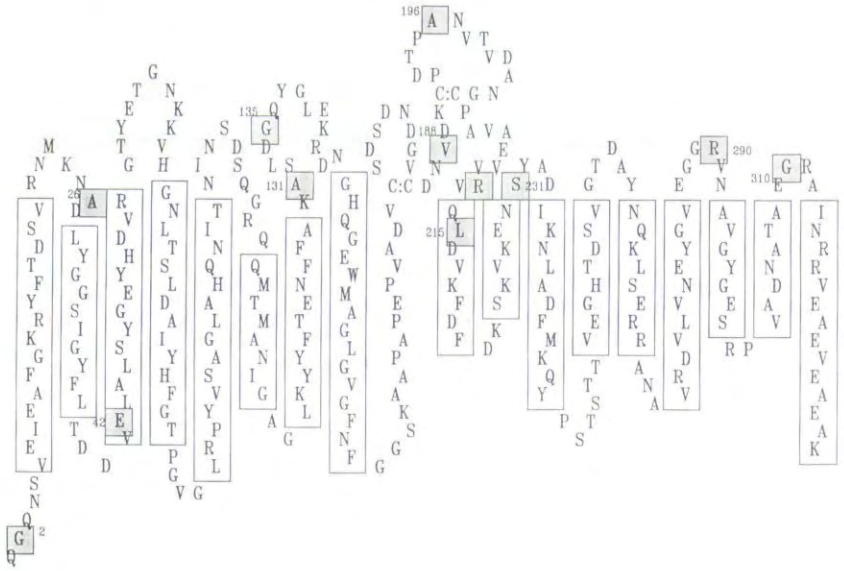

Fig. 5. Proposed membrane topology of OprF. The insertion sites in the linker mutants are marked by shaded boxes.

previously demonstrated to recognize overlapping but discrete epitopes within the cysteine disulphide region of OprF, a result consistent with the data obtained for the OprF variants produced by pRW307 and pRW308. The other four insertion sites did not interrupt specific monoclonal antibody-binding sites but were placed on the surface in keeping with the PhoE precedent discussed above and on the basis of preliminary information derived from the insertion of a malarial epitope into these sites (R. S. Y. Wong and R. E. W. Hancock, unpublished). The second criterion utilized for the model in Fig. 5 was based on the alignment of the *oprF* genes from *P. aeruginosa, Pseudomonas syringae* and *Pseudomonas fluorescens* (de Mot *et al.,* 1991). Since it is the surface regions of outer membrane proteins that undergo maximal variation (Sikkema and Murphy, 1992), it was considered that the non-homologous regions and/or small deletions should be preferentially located at surface loops. The third criterion was prediction of flexible segments by the Nowotny method using the computer program PC GENE. These flexible segments were considered to be candidates for loop regions. The fourth criterion was the prediction from circular dichroism of 62% β-sheet in OprF (Siehnel *et al.,* 1990). In fact the resultant model in Fig. 5 contains 56% β-sheet, and 61% if the central transmembrane domain is considered to be β-sheet. Finally, we included two disulphide bonds, a result consistent with 2-mercaptoethanol titration studies (Hancock and Carey, 1979) and, based on previous molecular genetic studies (Finnen *et al.,* 1992), placed the first disulphide between the first pair of cysteines. While having some similarities to the OmpF-like porin structural models, we have drawn it as a two-domain structure since, in the interconnecting regions of *E. coli* OmpA protein and *P. fluorescens* OprF, there are large proline, alanine repeats that are reminiscent of hinge regions. In *P. aeruginosa* and *P. syringae* OprF the cysteine loop region has apparently been inserted at this region, but we felt that the 'hinge' concept should be retained.

The topological model in Fig. 5 will provide the basis for future testing. Having an understanding of how insertions at different sites affect the structure and stability of the protein, it will be interesting to study the influence of the insertions on the porin function of OprF.

Experimental procedures

Bacterial strains and plasmids

E. coli strain DH5αF'φ80d*lacZ*ΔM15Δ(*lacZYA–arg*F)U169 *endA1 recA1 hsdR17*($r_k^-m_k^-$)*deo*R *thi-1 supE44* λ$^-$*gyrA96 relA1*) was used as the host for transformations and as the background strain for the expression of plasmids encoding the modified OprF proteins unless otherwise stated. The plasmid pUC4KAPA in a background strain *E. coli* HB101 (F$^-$*supE44 hsdS20*($r_B^-m_B^-$)*recA-13 ara-14 proA2 lacY1 galK2 rpsL20 xyl5 mtl-1*) was obtained from Dr J. Smit (University of British Columbia, Canada). It contained a 1.3 kb fragment, derived from Tn*901*, which encoded the enzyme aminoglycoside 3'-phosphotransferase (conferring kanamycin resistance), and was flanked by symmetrical restriction enzyme-recognition sites. For indirect immunofluorescence labelling and trypsinization studies, *E. coli* strain C386 (*lpp*, *ompA*) (Woodruff and Hancock, 1989) was used as the background strain in some of the experiments for the expression of modified OprF from plasmids. When plasmids were present, media containing 75 μg ml^{-1} of ampicillin or 50 μg ml^{-1} each of kanamycin and ampicillin were used.

The OprF-encoding plasmid for linker mutagenesis was constructed as follows. A *Pst*I–*Sal*I fragment from plasmid

pWW2200 (Woodruff and Hancock, 1989) containing the upstream sequence and *N*-terminus of the *oprF* gene was cloned into the vector pRK404 and was mutated by site-directed mutagenesis using the Mutagene kit (BioRad) in conjunction with the mismatch primer 5-'AAGTTCTGATAAGCT-TGCCACCCAA-3'. This procedure introduced a *Hin*dIII site into the putative –10 region of the *oprF* promoter by adding a G:C nucleotide pair between nucleotides –9 and –10 (using the numbering of Duchene *et al.* (1988)). This had the effect of substantially weakening the *oprF* promoter. A 0.37 kb *Hin*dIII/*Sma*I fragment corresponding to the *N*-terminus one-third of OprF was cloned into pTZ18R to construct pHJ12. A 4.5 kb *Sma*I fragment from pWW13 (Woodruff *et al.*, 1986) containing the rest of the *oprF* gene was then cloned into the *Sma*I site of pHJ12 to construct pHJ14. The 1.45 kb *Hin*dIII/*Kpn*I partial fragment containing the entire *oprF* gene from pHJ14 was then subcloned into the vector pTZ19R to obtain pRW3 (Fig. 1).

Linker-insertion mutagenesis

The plasmid pRW3 was linearized separately by partial digestion with restriction enzymes: *Rsa*I, *Hae*III, *Tha*I or *Alu*I (Boehringer Mannheim), all of which leave blunt ends after digestion. In the cases of *Alu*I and *Tha*I, partial digestions were performed in the presence of ethidium bromide (20 and 50 $\mu g\,ml^{-1}$ respectively) to improve chances of the recovery of DNA molecules cut at a single site. After partial digestions, the reaction mixtures were loaded onto preparative agarose gels and the full-sized linear form of the plasmid was isolated by elution onto DEAE paper (Schleicher and Schuell). The four pools of linearized pRW3, each corresponding to a separate restriction enzyme used, were ligated separately with a 1.3 kb *Hin*cII fragment containing the kanamycin-resistance cassette from pUC4KAPA. Following ligation and transformation, cells were plated on Luria agar plates containing 50 $\mu g\,ml^{-1}$ each of kanamycin and ampicillin. The doubly resistant colonies were further screened on colony immunoblots for loss of expression of OprF using the monoclonal antibody MA5-8, which is specific for the *C*-terminus of OprF (Finnen *et al.*, 1992). Plasmid DNA from the clones that did not express OprF was isolated by the alkaline lysis method (Sambrook *et al.*, 1989). The extracted plasmid DNA from each clone was then digested with *Pst*I, which only recognized sites in the flanking sequences of the kanamycin-resistance cassette, and hence cleaved the cassette from the plasmid. Following re-ligation of the *Pst*I-digestion mixtures and transformation, cells were plated on ampicillin-containing medium. Colonies which appeared were screened for the recovery of both immunoreactive OprF, using monoclonal antibodies and colony immunoblots (Mutharia and Hancock, 1985), and kanamycin sensitivity. The OprF-expressing, kanamycin-sensitive clones presumably contained mutated forms of pRW3 with a 12 bp insertion at sites originally interrupted by the kanamycin-resistance cassette. Plasmid DNA was prepared from these clones and the insertion sites were mapped by restriction pattern analysis by double digestion with *Pst*I/*Hin*dIII and *Pst*I/*Sal*I, where *Hin*dIII and *Sal*I were unique sites outside and within the *oprF* gene coding sequence, respectively. Clones with the same restriction pattern were grouped and at least one representative from each group was chosen for further analyses.

Construction of pRW307

The plasmid pRW307 was constructed by inserting, into the *Sal*I site corresponding to amino acid 188 in the OprF sequence, a self-hybridizing *Sal*I adaptor oligonucleotide (5'-TCGACCTGCAGG-3') which carried a *Pst*I site. As a result, the four amino acids DLQV were added after the valine residue at position 188.

DNA sequencing

DNA sequencing was performed to determine the exact position of the insertion sites of the representative linker-insertion mutants. Plasmid DNA from the corresponding kanamycin-resistant clones were used as templates. Template DNA was prepared using Qiagen columns (Qiagen Inc.) according to the manufacturer's protocols. The sequencing primers used were 25-mer oligonucleotides (5'-ATGTAACATCAGAGATTTTGA-3' and 5'-TATGAGTCAGCAACACCTTCT-3') that hybridized to opposite strands of the kanamycin-resistance cassette, approximately 50 bp from the ends of the cassette (Oka *et al.*, 1981). Automated DNA sequencing was carried out with the Applied Biosystems Incorporated (ABI) model 373A DNA sequencing system using polymerase chain reaction and dye-terminator chemistry as described in ABI's protocols. Sequence analyses were performed using the ABI 675 DNA sequence editor program.

Characterization of outer membranes

E. coli strains containing the pRW3-derived plasmids were grown overnight at 37°C in Luria broth supplemented with 75 $\mu g\,ml^{-1}$ ampicillin and 1 mM IPTG. Outer membrane samples were isolated by selective Triton X-100 solubilization of cell envelopes (Schnaitman, 1971). The final outer membrane pellet from 50 ml of cells was resuspended in 500 µl of distilled water. SDS–PAGE (Hancock and Carey, 1979) and Western immunoblotting (Mutharia and Hancock, 1985) utilized the procedures described previously, loading respectively 16 and 8 µg of outer membrane proteins into each lane. The OprF-specific monoclonal antibodies used in these analyses were described previously (Finnen *et al.*, 1992).

Trypsinization studies

E. coli strain C386 containing various pRW3-derived plasmids were grown in Luria broth supplemented with 75 $\mu g\,ml^{-1}$ of ampicillin to an A_{600} of 0.8. Samples of 1.5 ml of the cultures were harvested and washed twice with 20 mM Tris-HCl pH 7.4 containing 5 mM $MgCl_2$. The cell pellets were resuspended in 500 µl of the same buffer. Trypsin (TPCK treated, purchased from Sigma Chemicals) was added at a final concentration of 0.1 $mg\,ml^{-1}$ of cell resuspension, followed by incubation at 37°C for 60 min. Untreated samples were incubated in the same conditions, except trypsin was omitted. Proteolysis was stopped by heating at 88°C for 10 min in solubilization–reduction mix (Hancock and Carey, 1979). OprF in outer membrane samples was digested at a trypsin concentration of 0.1 $mg\,ml^{-1}$. The reactions were carried out as described above. In both cases, the trypsinized samples were run on SDS–polyacrylamide gels and analysed by Western immunoblotting with the

specified monoclonal antibodies. As controls, the cleavage of OprF in *P. aeruginosa* intact cells to a 28 kDa core fragment and the complete cleavage of bovine serum albumin by trypsin to small-molecular-weight peptides were demonstrated.

Indirect immunofluorescence labelling of intact cells

Immunofluorescence labelling was performed as follows. Briefly, overnight cultures of strains containing the specified plasmids were harvested and washed twice in PBS. Slides were coated with poly-L-lysine (Sigma Chemical Co.; average molecular weight ~25 000) by flooding with poly-L-lysine solution (1 mg ml^{-1}) in a moist chamber for 15–20 min and then rinsed thoroughly with distilled water. Samples of washed cells were smeared onto the poly-L-lysine coated slides and allowed to air dry briefly. Slides were then incubated with OprF-specific monoclonal antibodies in a 1/100 dilution in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) for 30 min at room temperature. After washing with excess PBS, slides were incubated with 1/20 dilutions of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (BRL) in PBS/1% FCS for 30 min at room temperature. Following PBS washings, one drop of Sigma mounting medium was added to the slides and the cells were examined under a Zeiss microscope fitted with a halogen lamp, a condenser and filters for fluorescence microscopy at 525 nm for emission of fluorescein isothiocyanate.

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