Chapter 20

Function and Structure of *Pseudomonas aeruginosa* Outer Membrane Protein OprF

Francis Bellido, Renee L. Finnen, Nancy L. Martin, Richard J. Siehnel, and Robert E. W. Hancock

NEW FUNCTIONAL ASPECTS OF OprF

Protein F (OprF) is a major outer membrane protein of *Pseudomonas aeruginosa* (Nikaido and Hancock, 1986). OprF has two main functions: in the permeability of the *P. aeruginosa* outer membrane as a channel-forming protein (porin) and also in the maintenance of the bacterial cell shape as a structural protein. However, as discussed in recent reviews (Hancock et al., 1990; Siehnel et al., 1990), the channel size and the nature of the OprF pore are actually disputed. Using different reconstituted model membrane systems, earlier studies (Hancock et al., 1979) had shown that the *P. aeruginosa* outer membrane exhibits a large apparent exclusion limit (3,000 Da). These results appeared to be at odds with reports pointing out that the permeability of the *P. aeruginosa* outer membrane was intrinsically low (Yoshimura and Nikaido, 1982; Angus et al., 1982), a property that would naturally decrease the penetration of harmful substances into cells and thus explain the high resistance of *P. aeruginosa* to antibiotics. Woodruff et al. (1986) found that such apparently contradictory data were in accordance with the observation that OprF might function as a heterogeneous porin. A small proportion (<1%) of the total OprF protein molecules formed large channels in black lipid bilayer studies, while the bulk of the reconstituted molecules formed small, apparently antibiotic-impermeable pores. Yoshimura et al. (1983) presented liposome swelling data in accordance with this hypothesis. In contrast, Nakae and collaborators recently released conflicting data (Yoneyama et al., 1986; Gotoh et al., 1989; Yoshihara and Nakae, 1989) based on the same types of liposome swelling assays. Briefly, their findings suggested that the exclusion limit of the *P. aeruginosa* outer membrane was much lower than previously reported. Furthermore, their results indicated that OprF did not function as a porin. Part of this controversy may come from the nature of the artificial model systems that have been used to study the permeability function of outer membrane proteins. Indeed, data generated by using these methodologies could be easily biased by technical limitations (see Hancock [1986] for a discussion).

Francis Bellido, Renee L. Finnen, Nancy L. Martin, Richard J. Siehnel, and Robert E. W. Hancock • Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3.

Consistent with this idea, Nikaido et al. (1991) have recently suggested that the purification method used by Nakae and collaborators may have inadvertently inactivated OprF. We felt that it was worthwhile to try a new methodological approach not based on model membrane studies. Therefore, we constructed mutants by insertion of either transposon Tn*1* or the interposon Ω into the cloned *oprF* gene, followed by gene replacement of the native *oprF* gene in *P. aeruginosa* with these disrupted *oprF* genes. Although small to marginal changes in antibiotic susceptibility were observed (Woodruff and Hancock, 1988), these results were not definitive since loss of OprF by mutation caused profound structural alterations, including enhanced hydrophobic uptake (Woodruff and Hancock, 1988), spherical morphology, and inability to grow in low-osmolarity medium (Woodruff and Hancock, 1989). Thus, any decreases in antibiotic uptake due to loss of OprF porin could be potentially balanced by enhanced uptake through the structurally altered outer membrane (Woodruff and Hancock, 1988). While β-lactam antibiotics do have some intrinsic ability to cross the outer membrane via non-porin routes (Hancock and Bell, 1988), we felt that sugars as large as di- to trisaccharides would pass only through water-filled pores. In this chapter, we summarize the development of an in vivo model based on the utilization of the *Escherichia coli* raffinose operon to study the passage of oligosaccharides across the outer membrane of *P. aeruginosa*.

P. aeruginosa can normally utilize only monosaccharides as a carbon source (Palleroni, 1984). Its inability to grow on higher-molecular-weight oligosaccharides may come from the lack of adequate metabolic pathways, since related species, such as *P. putida* and *P. syringae*, can sometimes grow on raffinose. Conversely, the low intrinsic permeability of the *P. aeruginosa* outer membrane might allow only monosaccharides to penetrate into the bacterial

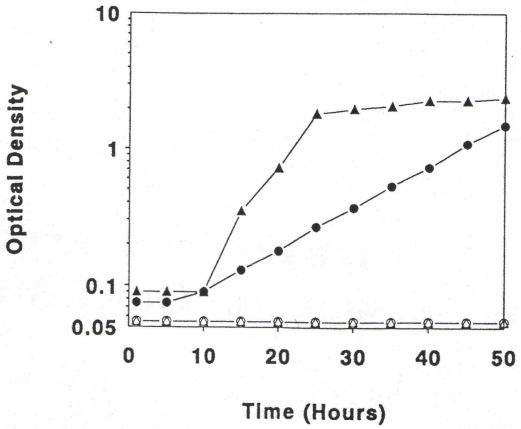

FIGURE 1. Growth curves of the wild-type strain H103 (△), the OprF-deficient insertion mutant H636 (○), and their derivatives H103(pFB15) (▲) and H636(pFB15) (●), containing the raffinose operon, on BM2 minimal medium supplemented with a 30 mM concentration of the disaccharide melibiose. H103 (pFB15) grows more than threefold faster than H636 (pFB15), whereas H103 and H636 were unable to use melibiose as a carbon source. The data are selected from Bellido et al. (submitted).

periplasm, as suggested by the experiments of Nakae and collaborators (Yoneyama et al., 1986; Yoshihara and Nakae, 1989). To address this question, the *E. coli* raffinose operon (Aslanidis et al., 1989) was cloned into the broad-host-range vector pVDtac 39 and the resultant plasmid, pFB15, was transferred to several strains of *P. aeruginosa* (Bellido et al., submitted). The three genes (those for α-galactosidase, cytoplasmic membrane permease, and sucrose hydrolase) encoded by the raffinose operon would potentially provide *P. aeruginosa* with the metabolic tools to utilize the disaccharide melibiose, the trisaccharide raffinose, and the tetrasaccharide stachyose as carbon sources. This strategy thus allowed an in vivo assessment of the exclusion limit of the *P. aeruginosa* outer membrane. Figure 1 shows that *P. aeruginosa* H103 (pFB15) grew efficiently in minimal medium supplemented by 1% melibiose. However, when raffinose was the sole carbon source, the growth was very slow. In contrast, the

OprF-deficient strain H636(pFB15) grew very slowly on melibiose and not at all on raffinose. H103(pFB15) and H636(pFB15) grew at similar rates on gluconate as a carbon source, indicating that these data did not reflect a general growth defect. These preliminary results suggest that the apparent exclusion limit of *P. aeruginosa* outer membrane is at least greater than the molecular size of raffinose, i.e., 500 Da. In addition, they strongly support the idea that OprF has a significant pore-forming activity for saccharides.

NEW STRUCTURAL ASPECTS OF OprF

Two studies have pointed out the analogy between OprF and the integral outer membrane protein of *E. coli*, OmpA (Duchêne et al., 1988; Woodruff and Hancock, 1989). For example, the carboxy-terminal half of OprF possesses significant amino acid homology to the same region in OmpA from *E. coli* or from other members of the family *Enterobacteriaceae* and pIII from *Neisseria gonorrhoeae* (Gotschlich et al., 1987). It has been proposed that in OmpA, the carboxy half of the protein rests in the periplasm (Morona et al., 1984; Klose et al., 1988; Vogel and Jähnig, 1986). However, when extrapolated to OprF, this model does not adequately explain the properties of this protein. In this review, we summarize data supporting a previously published structural model for OprF whereby portions of the C-terminal half of the protein are exposed to the outside surface of the cell (Siehnel et al., 1990).

The structure of a protein must reflect its functional capabilities; therefore, a structural model of OprF should include features which allow it to form a pore and also to contribute toward cell shape. We previously published a model for OprF (Siehnel et al., 1990) using a β-turn predictive method which accommodated several pieces of information: (i) circular dichroism shows OprF to contain 62% β-sheet structure, even after extensive heating (Martin et al., submitted), and a high content of β-sheet structure has been demonstrated in several outer membrane proteins which are porins; (ii) OprF has at least one, and probably two, disulfide bonds; and (iii) OprF has no long stretches of hydrophobic amino acids which would readily embed in a phospholipid membrane. Recently, we have used a series of 10 monoclonal antibodies specific for OprF to further define the structural features of OprF (Finnen et al., submitted; Martin et al., submitted). Two different approaches have been used to isolate specific regions of OprF which would subsequently allow us to map the epitopes recognized by this bank of monoclonal antibodies. One approach was to make a series of fusion proteins consisting of a truncated OprF region of various lengths, using Tn*phoA* mutagenesis (Finnen et al., submitted). A second approach involved generating OprF peptides by proteolysis and cyanogen bromide cleavage (Martin et al., in preparation).

The Tn*phoA* mutagenesis system developed by Manoil and Beckwith (1985) has been applied by researchers to study the topology of both cytoplasmic membrane proteins (Lloyd and Kadner, 1990; Winans et al., 1989; Herrero et al., 1988) and outer membrane proteins (Murphy et al., 1990). In our case, a collection of OprF fusion proteins was established by infecting, with λ Tn*phoA*, *E. coli* TB1 carrying the *P. aeruginosa oprF* gene on plasmid pWW2200. Successful transpositions were selected by tetracycline and kanamycin resistance. To separate successful transpositions onto plasmid DNA from chromosomal transposon mutants, plasmid DNA was isolated from the pool of doubly resistant colonies, and the pooled plasmid preparation was used to transform an alkaline phosphatase (PhoA)-deficient strain of *E. coli*, CC118. Transformants were again selected for tetracycline resistance (to select for plasmid maintenance) and kanamycin

resistance (to select for transposon insertions) and screened for production of functional alkaline phosphatase by the inclusion of 5-bromo-4-chloro-3-indolyl phosphate in the agar medium. Both PhoA-positive and -negative clones were selected for further study (Finnen et al., submitted).

Tn*phoA* mutagenesis of the OprF gene resulted in a variety of fusion proteins. Depending on the orientation and reading frame of the insertion, the fusions proteins due to Tn*phoA* insertions at different sites were predicted by DNA sequencing to consist of a truncated OprF region fused either to PhoA or to peptides of 1 to 20 amino acids in length derived from the flanking IS*50* elements of the Tn*phoA* insert. Most of the alkaline phosphatase-producing fusions occurred at fusion joints in the first 150 amino acids and were not detectable by our monoclonal antibodies. Only fusions occurring at OprF amino acids 204, 289, and 299 (Fig. 2) gave rise to a product that was immunodetectable on Western immunoblots with OprF-specific monoclonal antibodies. The reactivities of these fusion proteins with the bank of anti-OprF monoclonal antibodies are shown in Table 1. Analysis of sequence data predicted that the fusion protein arising from the insertion at position 204 would lack the second disulfide bridge in the model (Siehnel et al., 1990). This finding indicated that only the first disulfide bridge was required for the formation of the epitopes recognized by monoclonal antibodies 4-4 and 7-8. These epitopes are not recognized when native OprF is treated with reducing agents such as β-mercaptoethanol (Mutharia and Hancock, 1985). In the case of fusions at 289 and 299, the major immunodetectable product was smaller than the predicted fusion protein, and full-size fusion proteins were not easily visualized. We feel that this is due to proteolytic digestion of presumably unstable fusion proteins, resulting in a typical, protease-resistant OprF peptide (Mutharia and Hancock, 1985; Woodruff et al., 1986). Even though the exact nature of these degradation products is not known, it can still be concluded that C-terminal sequences of OprF are required for the formation of some surface epitopes. Consistent with this view, pWW1602, which contains the cloned carboxy-terminal half of OprF, produced a protein that reacted with 7 of the 10 monoclonal antibodies (Fig. 2; Table 1). These monoclonal antibodies had been isolated after immunization with intact protein F.

The regions of the epitopes recognized by the 10 monoclonal antibodies were also determined after chemical cleavage and proteolysis of purified OprF. The OprF peptides generated by cyanogen bromide or protease digestion were analyzed by Western immunoblotting with the anti-OprF monoclonal antibodies. The reactive peptides were then identified by N-terminal sequencing and amino acid analysis. Figure 2 shows some of these peptides, and Table 1 summarizes their reactivities with the tested antibodies. Most of the antibodies appeared to be directed toward epitopes in the C-terminal region of OprF.

Immunofluorescent labeling of intact *P. aeruginosa* cells was carried out to determine whether any of the epitopes recognized by the monoclonal antibodies were located on the cell surface. Monoclonal antibodies 7-5, 4-4, and 5-8 all reacted strongly with whole cells, indicating that these epitopes are exposed at the surface of the cell. Our mapping data (Table 1) show the epitopes for monoclonal antibodies 7-5 and 5-8 to be in the C-terminal portion of OprF. Overall, these data favor the hypothesis that the carboxy terminus of OprF, i.e., that region most similar to OmpA (Woodruff and Hancock, 1989) is at least in part cell surface exposed, and they support in general a previously published structural model for OprF (Siehnel et al., 1990).

SUMMARY

Although OprF has been studied extensively, many of its functional and structural

FIGURE 2. Schematic representation of OprF peptides. Peptides 1, 2, and 3 were generated by protease digestion with papain. The sizes of these peptides were estimated from amino acid analyses; therefore, the approximate C termini are indicated by dashed lines. CnBr peptide was generated by cyanogen bromide cleavage. pWW1602 is an OprF subclone expressing only amino acids 190 to 326. Fusion 204 was a truncated OprF peptide produced by Tn*phoA* fusion. The fusion joint at amino acid 204 occurred between the pairs of cysteines indicated by C above OprF. Fusion proteins 289 and 299 were predicted from DNA sequencing to contain 289 and 299 amino acids, respectively, of OprF. However, on Western immunoblots they were observed to produce a predominant product similar to that of peptide 2. These results are selected from those of Finnen et al. (submitted) and Martin et al. (submitted).

TABLE 1
Monoclonal antibody activities against various OprF-derived peptides

Peptide	Monoclonal antibody reactivity[a]					
	7-1	7-8, 4-4	7-4	7-2, 7-6	7-3, 7-5, 7-7	5-8
Peptide 1	+	+	+	+	−	−
Peptide 2	+	+	−	−	−	−
Peptide 3	−	−	−	+	−[b]	+
CnBr peptide	ND	−	ND	ND	ND	+
pWW1602	−	−	+	+	+	+
Fusion 204	+	+	−	−	−	−
Fusion 289/299	+	+	−	+/−	−	−

[a] The monoclonal antibodies are grouped according to their apparent epitope locations, 7-1 reacting with the N-terminal area and 5-8 reacting with the C-terminal area of OprF. +, reactive; −, non-reactive, +/−, weakly reactive; ND, not determined.

[b] Monoclonal antibodies 7-3, 7-5, and 7-7 did not react with peptide 3 generated by papain digestion, despite the fact that this peptide appeared to overlap the region of OprF containing the epitopes for these monoclonal antibodies. We assume this lack of reactivity is due to an inappropriate configuration adopted by this peptide or the requirement for amino acids in the region of amino acid 200.

aspects remain unclear. Thus, the role of OprF as a porin is currently a source of controversy. To address this issue, the *E. coli* raffinose operon was cloned and expressed in *P. aeruginosa*. This methodology allowed us to study the influence of outer membrane proteins in the passage of different-molecular-weight polysaccharides across the outer membrane. The results obtained so far suggest that OprF has a significant pore-forming activity providing *P. aeruginosa* outer membrane with an exclusion limit of greater than 500 Da.

Several experimental approaches were carried out to determine the surface features of OprF. By examining the monoclonal antibody reactivity patterns of various OprF peptides and OprF-Tn*phoA* fusion proteins, we were able to localize several epitopes, some of which are surface exposed and located in the C-terminal portion of OprF. Contrary to what has been proposed for OmpA, a related protein in *E. coli*, our results suggest that regions of the C-terminal portion of OprF are exposed on the external surface of the outer membrane.

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