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Epitope Mapping of the *Pseudomonas aeruginosa* Major Outer Membrane Porin Protein OprF

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The *Pseudomonas aeruginosa* major outer membrane protein OprF has been proposed for use as a vaccine and as a target for immunotherapeutic and diagnostic monoclonal antibodies. The well-conserved epitopes for 10 surface-reactive, OprF-specific monoclonal antibodies were localized by both overlapping peptide analysis and immunodetection of OprF peptides generated by cyanogen bromide and the protease papain. Three of the monoclonal antibodies bound to specific overlapping octapeptides, which had been synthesized on 160 pins to cover the entire 326 amino acids of OprF. The highest reactivities were as follows: MA7-1 to the pin with attached peptide GTYETGNK (amino acids 55 to 62), MA7-2 to NLADFMKQ (amino acids 237 to 244), and MA5-8 to TAEGRAIN (amino acids 307 to 314). The other monoclonal antibodies showed no reactivity, indicating that they do not recognize linear epitopes. Two polyclonal sera were also tested and demonstrated weak reactivity with discrete regions of OprF, suggesting that the majority of antibodies produced might recognize conformational epitopes. Utilizing defined peptides generated with cyanogen bromide and papain, the conformational epitopes recognized by the seven monoclonal antibodies were localized to regions that were 42 to 90 amino acids long. These regions were located on two adjacent loops in the middle of an amended structural model of OprF.

OprF, the major porin protein in *Pseudomonas aeruginosa*, forms channels across the outer membrane for di- and trisaccharides (1). OprF-deficient mutants have rounded morphology and grow well only in high-osmolarity media (9, 18), suggesting that OprF has a role in determination of outer membrane and cell structure and stability. Although OprF is a porin, its structure is probably quite different from that of the majority of bacterial porins (12). Analyses of amino acid sequence homology have suggested that OprF belongs to a class of proteins which are proposed to have a wide range of functions (3). This class includes diverse proteins such as MotB, a cytoplasmic membrane protein which is part of the flagellar rotation assembly in *Escherichia coli* and *Bacillus subtilis*, and YFIB, which is a lipoprotein (3). Interestingly, it is only the C-terminal portions of these proteins which show any sequence homology, suggesting a common function for this portion of the protein.

As OprF is an abundant protein in *P. aeruginosa*, it presents an attractive target for diagnosis and treatment of *Pseudomonas* infections. In studies utilizing monoclonal antibodies MA7-3 and MA4-4, specific for OprF, it has been demonstrated that this protein is potentially useful as a target for diagnostic and immunotherapeutic intervention (2, 11). Indeed, Gilleland and colleagues (8) have presented data to demonstrate that immunization with OprF protects against subsequent *P. aeruginosa* infections.

In a previous study, we described 10 anti-OprF monoclonal antibodies and localized their epitopes using truncated forms of OprF, including Tn*phoA* derivatives (5). Unfortunately, the epitopes for most of the antibodies could be only crudely localized by this means, and it is not known to what extent the structure of recombinant C-terminal deletion derivatives reflects the structure of the native protein. In this study, we have further characterized these epitopes using both overlapping peptide analysis and cyanogen bromide- and protease-cleaved peptides of purified OprF.

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MATERIALS AND METHODS

Peptide synthesis and epitope scanning. Support-coupled overlapping octapeptides starting at every second amino acid position of OprF were purchased from Chiron Mimotopes, Clayton, Australia. The peptides attached to polyamide pins in arrays of 96 pins were used as antigens in enzyme-linked immunosorbent assay (ELISA) studies in which the pins were inserted into ELISA plates as described previously (7). The ELISAs were performed, in two independent trials, as described in Geysen et al. (7), except that the absorbance was assessed periodically to ensure that readings were in the linear range of the machine. Positive and negative controls supplied with the kit were performed (data not shown). Antibodies were used at dilutions of 1/10,000 to 1/2,500 for monoclonal antibodies and 1/5,000, 1/500, or 1/350 for polyclonal antibodies.

Purification of OprF. *P. aeruginosa* JYH4, an OprL-deficient environmental isolate (10), was grown in Mueller-Hinton broth with shaking for 5 to 6 h at 37°C, harvested by centrifugation, and suspended in 10 mM Tris-HCl, pH 8.0, containing 20% (wt/vol) sucrose and 50 μg of DNase per ml. The cells were broken by passage through a French pressure cell two or three times at 14,000 lb/in^2, and the unbroken cells were removed by centrifugation at 3,000 × *g* for 10 min. Cell envelopes were collected by centrifugation at 100,000 × *g* for 1 h, suspended in 15 mM Tris-HCl, pH 8.0, which contained 2% (wt/vol) Zwittergent 3-14, and centrifuged again. The detergent-insoluble fraction was suspended in 15 mM Tris-HCl (pH 8.0)–2% Zwittergent 3-14–1 M NaCl. This suspension was incubated with gentle shaking at 37°C overnight and then centrifuged as above. The supernatant, which contained OprF, was desalted by passage over a spun column of Sephadex G-25 (Bio-Rad, Hercules, Calif.), incubated with 0.1 mg of lysozyme per ml for 1 h, then applied to a Sepharose 4B (Bio-Rad) column, and eluted with 15 mM Tris-HCl, pH 8.0, which contained 0.5% Zwittergent 3-14 and 0.4 M NaCl. Peaks which contained OprF were pooled, loaded onto a fast protein liquid chromatography (FPLC) gel-sieving column (Superose-12; Pharmacia, Piscataway, N.J.), and eluted with 15 mM Tris-HCl (pH 8.0)–0.1% Zwittergent 3-14–0.4 M NaCl.

The samples which contained OprF were desalted as described above, dialyzed for 48 h against 10 mM Tris-HCl (pH 8.0)–0.1% Zwittergent 3-14, then applied to an FPLC anion-exchange column (Mono-Q; Pharmacia) in 10 mM Tris-HCl (pH 8.0)–0.1% Zwittergent 3-14–10% methanol, and eluted with a linear gradient of 0 to 1.0 M NaCl in the same buffer. OprF was eluted in a single peak.

Protease digestion. Purified OprF was subjected to enzymatic cleavage with papain (papainase type 4; Sigma, St. Louis, Mo.) in 2 mM EDTA–20 mM Tris-HCl (pH 6.0) at a concentration of 50 μg of enzyme per mg of protein for 18 h at 4°C. At the end of the digestion time, the sample was precipitated with

ice-cold ethanol, and the pellet was suspended in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted either onto polyvinylidene difluoride membranes (14) for amino acid analysis and N-terminal sequencing or onto nitrocellulose for Western blotting (immunoblotting) (15).

Cyanogen bromide cleavage. Cleavage of OprF by cyanogen bromide followed the method of Garten and Henning (6). One hundred micrograms of purified, lyophilized OprF was suspended in 1 ml of 78% formic acid containing 1 M cyanogen bromide and 0.6 N HCl. This was approximately a 65,000-fold molar excess of cyanogen bromide to methionine residues in OprF. The sample was tightly capped, covered with foil, incubated at room temperature for 18 h, and then diluted to 10 ml with deionized water and lyophilized. The dried sample was dissolved in 10 ml of deionized water and lyophilized again. The final dried sample was suspended in electrophoresis sample buffer, loaded, and run on a 14% acrylamide gel. The samples were then transferred to nitrocellulose for immunoblotting or to a polyvinylidene difluoride membrane for N-terminal sequencing.

Determination of the amino-terminal sequence and amino acid composition. Peptides were prepared for both N-terminal sequence analysis and amino acid composition determinations by the procedure of Matsudaira (14). After transfer of peptides to the polyvinylidene difluoride membrane, the membrane was air dried and the peptide bands were cut out and stored separately in sterile microcentrifuge tubes. Analyses were performed by S. Kielland at the Protein Microchemistry Centre (Department of Biochemistry and Microbiology, University of Victoria, Victoria, Canada), where a gas phase sequencer (model 470A; Applied Biosystems) was used for N-terminal analysis and the amino acid analysis was done on an Applied Biosystems 420A derivatizer-analyzer system after hydrolysis of the peptides by gas phase techniques.

OprF-specific antibodies. The OprF polyclonal rabbit serum was made by subcutaneously injecting 100 μg of FPLC-purified OprF, in Freund's complete adjuvant, into a New Zealand White rabbit. This was followed by two more injections, at 5-week intervals, in Freund's incomplete adjuvant. Serum was collected 3 days after the last injection. The polyclonal mouse serum was obtained by immunizing, intraperitoneally, BALB/c mice with 20 μg of FPLC-purified OprF in Hunter's Titremax, (Cedarlane Laboratories Ltd., Hornby, Canada). This was repeated twice at 2-week intervals. Serum was collected 3 weeks after the last injection. The monoclonal antibodies used were as described by Finnen et al. (5).

RESULTS

Mapping of epitopes with overlapping octapeptides. Overlapping octapeptides, starting at every second amino acid and covering the entire 326 amino acids (aa) of OprF were synthesized on 160 pins. ELISAs were performed with 10 monoclonal and 2 polyclonal anti-OprF antibodies. Reactivity was observed with only 3 of the 10 monoclonal antibodies, as shown in Fig. 1. MA7-1 bound to three pins derivatized with the peptides VRGTYETG (aa 53 to 60, pin 27), GTYETGNK (aa 55 to 62, pin 28), and YETGNKKV (aa 57 to 64, pin 29). The binding of MA7-1 to pin 28 was ~3 times that of pin 29 and 10 times that of pin 27. MA7-2 bound primarily to the peptide NLAD FMKQ (aa 237 to 244, pin 119) but did bind with low affinity to the peptide IKNLADFM (aa 235 to 242, pin 118). MA5-8 bound to the peptide with the sequence TAEGRAIN (aa 307 to 314, pin 154) with a three-times-greater affinity than to the peptide NATAEGRA (aa 305 to 312, pin 153). MA7-3, MA7-4, MA7-5, MA7-6, MA7-7, MA7-8, and MA4-4 showed no reactivity (data not shown), indicating that they do not recognize linear epitopes.

To examine the extent of distribution of linear epitopes in the OprF sequence, we tested polyclonal antibodies from pooled mouse and rabbit sera. The mouse serum was tested at a working dilution of 1/5,000 and again at a dilution of 1/350. The results were similar, and those for the 1/350 dilution are presented in Fig. 2A. The antibodies bound with high affinity to pins 153 and 154, which correspond to the same region as the epitope for MA5-8 and bound more weakly to peptides composed of aa 15 to 22 (pin 8), 281 to 288 (pin 141), and 295 to 306 (pins 148 to 150). This serum bound purified OprF in a standard ELISA when used at a dilution of 1/100,000 (16b) and specifically recognized OprF on immunoblots of whole-cell lysates of wild-type *P. aeruginosa* at a dilution of 1/1,000 (data not shown).

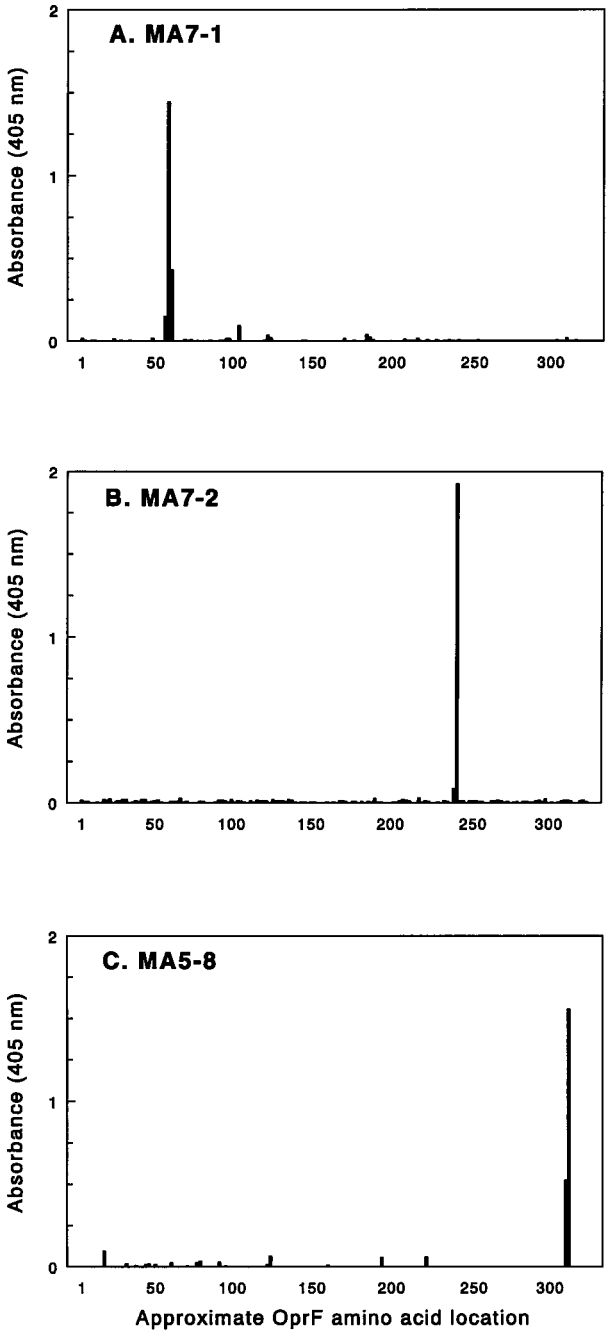

FIG. 1. ELISA readings of monoclonal antibodies MA7-1 (A), MA7-2 (B), and MA5-8 (C) reacting with individual pins derivatized with overlapping octapeptides from OprF. Background values were subtracted. The positions of the middle amino acids of the OprF-derived peptides, starting from the N terminus, are indicated on the *x* axis. These data are representative of two independent trials with essentially identical results.

No high-affinity antibodies were detected in the polyclonal rabbit serum when tested at a dilution of 1/500 (Fig. 2B). By increasing the development time, multiple weaker binding sites were observed. Of these sites, those that bound with an absorbance of at least 0.5 include peptides of aa 13 to 20 (pin 7), 83 to 90 (pin 42), 117 to 124 (pin 54), 127 to 134 (pin 59), 213 to

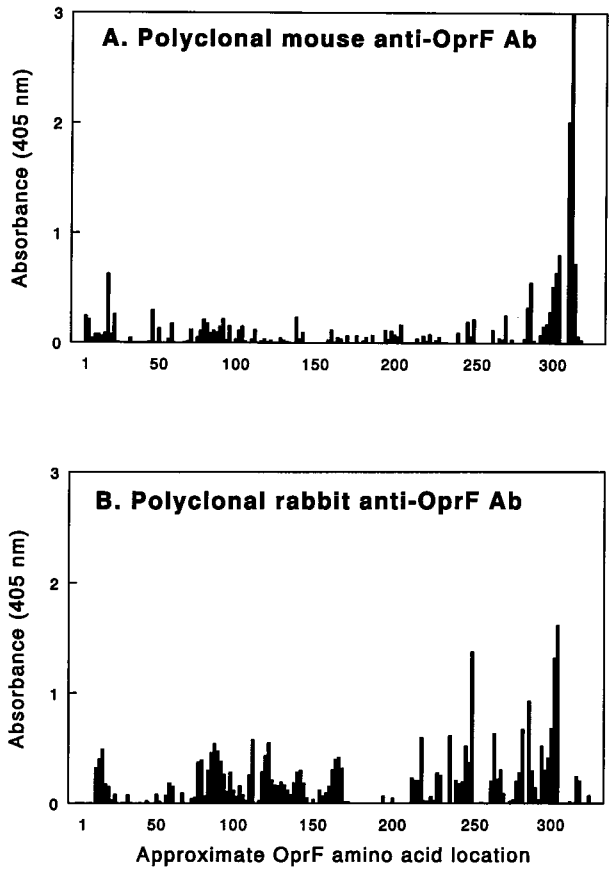

FIG. 2. ELISA readings of mouse (A) and rabbit (B) polyclonal sera (antibodies [Ab]) reacting with individual pins derivatized with overlapping octapeptides from OprF. Background values were subtracted. The positions of the middle amino acids of the OprF-derived peptides, starting from the N terminus, are indicated on the *x* axis. These data are representative of two independent trials with essentially identical results.

220 (pin 107), 231 to 238 (pin 116), 241 to 248 (pin 121), 259 to 266 (pin 130), 277 to 284 (pin 139), 281 to 288 (pin 141), and 289 to 296 (pin 145). Peptides of aa 245 to 252 (pin 123) and 295 to 306 (pins 149 and 150) had ~2- to 2.5-times-greater binding than the other binding sites. This serum bound specifically to OprF on immunoblots of whole-cell lysates of wild-type *P. aeruginosa* (16a) at a dilution of 1/500. The polyclonal sera appeared to share weak binding only to pins 141 and 149-150. These results suggest that, like the monoclonal antibodies, the majority of the polyclonal antibodies produced were to conformational epitopes.

Mapping epitopes with cyanogen bromide-cleaved and papain-digested peptides. Immunoreactivity of the 10 monoclonal antibodies with cyanogen bromide peptides was assessed by immunoblotting (Table 1). Peptides f2, f4, and f5 generated unique patterns of immunoreactivity. The cyanogen bromide fragments 1 and 3 comprising amino acids 1 to 23 and 110 to 151, respectively, were not observed in this study, and we assume that they ran at the dye front under the electrophoresis conditions used. However, the information presented in Table 1 and Fig. 1 indicated that they would not be immunoreactive with any of the monoclonal antibodies studied here and none of the monoclonal antibodies bound to a band at the dye front. Other peptides, f3-4, f4-5, and f3-4-5, comprising two or more cyanogen bromide fragments were also observed (Table 1). The generation by cyanogen bromide of peptides containing internal methionines, as observed in this study, has been previously described for OprF and other outer membrane proteins (6, 15). Peptide f3-4, comprising uncleaved fragments 3 and 4, had the same immunoreactivity pattern as peptide f4. This indicated that none of the monoclonal antibodies studied recognized an epitope within fragment 3. Peptide f4-5 had the immunoreactivities of the individual fragments f4 and f5 but, in addition, reacted with monoclonal antibodies MA7-2, MA7-3, and MA7-5, suggesting that the epitopes for these antibodies contained amino acids from both fragments. Data from the peptide mapping of MA7-2 (Fig. 1B) show that the epitope for this antibody straddles the junction of peptides f4 and f5, requiring aa 237 to 244 for high-affinity binding. Peptide f3-4-5 showed the same immunoreactivity as peptide f4-5, a result consistent with the lack of epitopes in fragment f3.

As an alternative procedure for generating peptides, OprF was digested with papain over a 24-h period to produce a total of 22 peptides, 8 of which were immunoreactive. The four most predominant immunoreactive peptides were subjected to amino-terminal sequencing and amino acid analysis. Interestingly, three of these contained the mature amino terminus of OprF

TABLE 1. Monoclonal antibody reactivity against OprF peptides generated by cyanogen bromide cleavage or by papain digestion

Peptide (aa)[a]	Reactivity[b]									
	MA4-4	MA5-8	MA7-1	MA7-2	MA7-3	MA7-4	MA7-5	MA7-6	MA7-7	MA7-8
Cyanogen bromide cleavage										
f2 (24–110)	−	−	+	−	−	−	−	−	−	−
f4 (152–242)	+	−	−	−	−	−	−	+	−	+
f5 (243–326)	−	+	−	−	−	−	−	−	−	−
f3-4 (113–242)	+	−	−	−	−	−	−	+	−	+
f4-5 (152–326)	+	+	−	+	+	−	+	+	−	+
f3-4-5 (113–326)	+	+	−	+	+	−	+	+	−	+
Papain digestion										
I (198–326)	ND	+	−	+	−	−	−	+	−	ND
II (1–210)	+	−	+	−	−	−	−	−	−	+
III (1–230)	ND	−	+	−	+	−	−	+	−	ND
IV (1–278)	ND	−	+	+	+	+	+	+	+	ND

[a] Amino termini of peptides f5, f3-4, f3-4-5, and I to IV were determined by N-terminal sequencing, and their carboxy termini were estimated from amino acid analyses and relative mobility of the peptides in SDS-PAGE. The identity of peptides f2, f4 and f4-5 was inferred from their relative mobilities in SDS-PAGE and their 2-mercaptoethanol modifiability (i.e., if modified by 2-mercaptoethanol, then the peptide contained the cysteine disulfide region). Since papain cleavage is not specific for particular amino acids, the carboxy termini of peptides I to IV are approximate.

[b] +, labelled on an immunoblot; −, not labelled; ND, not done. The data for peptides I to IV were obtained after SDS-PAGE in the presence of 2-mercaptoethanol. Untreated OprF reacted with all monoclonal antibodies.

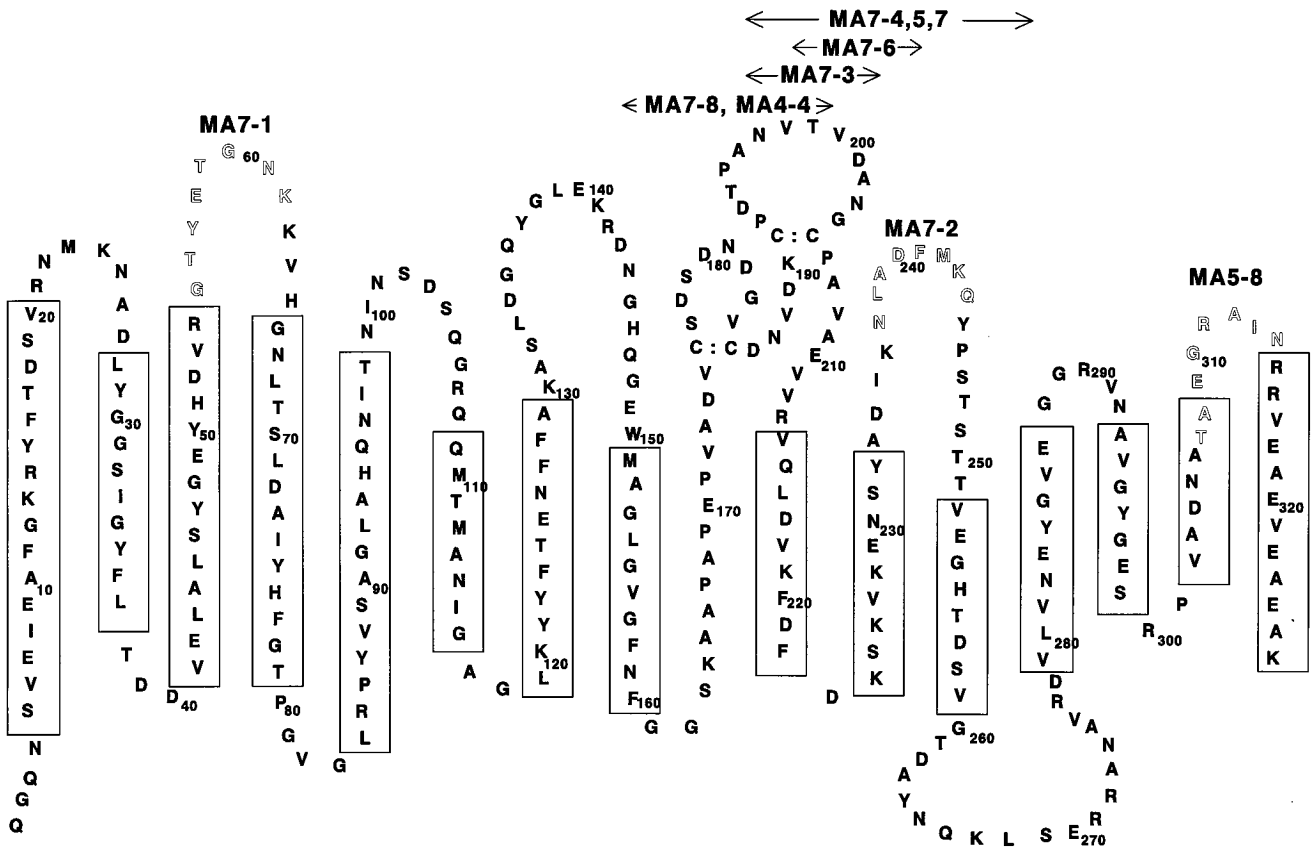

FIG. 3. Proposed secondary-structure model of OprF. The locations of epitopes are indicated by outlined letters and by arrows. The N terminus is on the bottom left-hand side of the model. Predicted regions of transmembrane β-strands are boxed.

(peptides II to IV). Indeed, this was the first protein sequence confirmation of the amino terminus of OprF, which was previously assumed to be blocked (4). One peptide (peptide I) began at aa 198 and extended to the carboxy terminus of the protein.

Immunoreactivity of the papain peptides was examined by immunoblotting (Table 1). Monoclonal antibody MA7-1 reacted with all peptides containing the amino terminus of OprF, whereas MA7-8 and MA4-4 reacted with all tested peptides that were not pretreated with 2-mercaptoethanol. Monoclonal antibodies MA7-3, MA7-4, MA7-5, and MA7-7 reacted only with peptides corresponding to at least 75% of the whole protein. The epitope for monoclonal antibody MA5-8 appeared to be close to the carboxy terminus of OprF since peptide I reacted with MA5-8 but peptide IV did not. Monoclonal antibodies MA7-2 and MA7-6 both reacted with peptides I and IV, suggesting that their epitopes were located after the cysteine region. However, the reactivity of MA7-6 but not MA7-2 with peptide III implied that these monoclonal antibodies had distinct epitopes, as confirmed by the overlapping peptide study above.

DISCUSSION

OprF, a major outer membrane protein of *P. aeruginosa*, is unusual among porins in that it appears to have several well-conserved surface epitopes (13, 16). Therefore, it was of interest to further define the nature of these epitopes, especially since they were expected to prove useful in refining the secondary-structure model of OprF. Overlapping peptide methodology allowed the localization of three of the OprF-specific monoclonal antibodies to regions of 8 aa. MA7-1 bound the peptide GTYETGNK with the greatest affinity. This region has been designated the second external loop in our proposed membrane topology map (Fig. 3). MA7-2 bound primarily to NLADFMQ, located in the sixth external loop, and MA5-8 bound to peptide TAEGRAIN, which, in our model, is a surface-exposed epitope located at the C-terminal end of the protein.

The remaining seven monoclonal antibodies appeared to be conformational in nature. The epitopes for MA4-4 and MA7-8 have been previously localized to the cysteine-containing region of OprF (references 11 and 5, respectively), and the peptide analysis presented here located them between aa 152 and 210. They would not be expected to react with linear peptides, since the epitopes for both of these proteins are rendered nonreactive in immunoblots by treatment with reducing agents (5). Linker insertion mutagenesis at aa 196 resulted in the loss of epitopes for both antibodies, while insertion at aa 188 resulted only in the loss of MA7-8 (17). This indicates that the antibodies bind separate epitopes. MA7-3 reacted with a papain-digested peptide containing aa 1 to 230, and, taking into consideration the results of Finnen et al. (5), the epitope can be located within aa 188 to 230. MA7-4, MA7-5, and MA7-7 reacted with papain-digested fragments containing a minimum of aa 1 to 278 (Table 1), and again, taking into consideration the results of Finnen et al., the region containing these epitopes can be localized to aa 188 to 278. MA7-6 bound cyanogen bromide-cleaved peptide f4, comprising aa 151 to 240, and could be further delineated to aa 198 to 240 on the

basis of reactivity with papain peptide I. The regions containing these epitopes are indicated in Fig. 3. The majority of these epitopes seem to be located within a two-loop region in the center of OprF. We presume that this region is highly structured and is stable even when released by cyanogen bromide or protease cleavage since (i) only one of the eight monoclonal antibodies mapping to this region recognizes a linear epitope, (ii) there were few linear epitopes in the first loop (aa 173 to 212) as judged by the interaction of polyclonal sera with the overlapping peptides (Fig. 2), and (iii) this region contains the disulfide bonds of OprF.

Our current model of OprF (Fig. 3), presented here, differs from the previously published version (17) in the C-terminal half. While the locations for the epitopes binding MA7-1 and MA5-8 in the previous model were consistent with surface epitope localization studies (13), that for MA7-2 was not. We have shown that, while the epitope for MA7-2 was not surface exposed in strains of *P. aeruginosa* M2 and H103, it was surface exposed in the rough lipopolysaccharide strain H692 and, therefore, could not be located entirely within the membrane. Furthermore, in contrast to our previous model, it seems likely that aa 213 to 221 form a transmembrane β-strand, as there is remarkable homology with the predicted β-strand 8. They both alternate the hydrophobic residues L, V, F, and F with hydrophilic residues. This pattern is highly conserved in the last strand of the porin superfamily (12). Another observation that is consistent with this model is that amino acids that are conserved in the OmpA family of porins (12) are predicted to be located primarily within the membrane. An exception is the highly conserved region from aa 262 to 273, which we have placed in the periplasm. This area is conserved between OmpA-related outer membrane proteins and MotB (which is not an outer membrane protein) of *B. subtilis* and has been proposed to be a site for peptidoglycan interaction (3). The results of this study are consistent with our proposal (5) that like the N-terminal region, the C-terminal region of OprF has surface-exposed loops and cannot, therefore, be located entirely in the periplasm as suggested for the homologous region of OmpA (see reference 5 for discussion). The precise location of epitopes described in this article will be useful for future structure-function studies.

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