FEMS Microbiology Letters 113 (1993) 261–266

Published by Elsevier

FEMSLE 05664

Conservation of surface epitopes in *Pseudomonas aeruginosa* outer membrane porin protein OprF

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(Received 28 June 1993; revision received 11 August 1993; accepted 17 August 1993)

Abstract: The outer membrane proteins of several prominent bacterial pathogens demonstrate substantial variation in their surface antigenic epitopes. To determine if this was also true for *Pseudomonas aeruginosa* outer membrane protein OprF, gene sequencing of a serotype 5 isolate was performed to permit comparison with the published serotype 12 *oprF* gene sequence. Only 16 nucleotide substitutions in the 1053 nucleotide coding region were observed; none of these changed the amino acid sequence. A panel of 10 monoclonal antibodies (mAbs) reacted with each of 46 *P. aeruginosa* strains representing all 17 serotype strains, 12 clinical isolates, 15 environmental isolates and 2 laboratory isolates. Between two and eight of these mAbs also reacted with proteins from representatives of the rRNA homology group I of the *Pseudomonadaceae*. Nine of the ten mAbs recognized surface antigenic epitopes as determined by indirect immunofluorescence techniques and their ability to opsonize *P. aeruginosa* for phagocytosis. These epitopes were partially masked by lipopolysaccharide side chains as revealed using a side chain-deficient mutant. It is concluded that OprF is a highly conserved protein with several conserved surface antigenic epitopes.

Key words: Porin; *pseudomonas aeruginosa*; Outer membrane; OprF; Epitope

Introduction

One of the strategies utilized by successful pathogens to evade the immune response is variation of surface antigen structure. For outer membrane proteins this can involve either mutations [1], or more complex genetic events such as rearrangements promoted by frameshifts [2]. The major outer membrane proteins of Gram negative bacteria have been shown, by either monoclonal antibody (mAb) reactivity or DNA sequence analysis to vary within individual strains of many species. Thus the outer membrane porin proteins from *Escherichia coli* [3], *Haemophilus influenzae* [4], *Chlamydia trachomatis* [5] and *Neisseria sp.* [6] have all been shown to have variations in surface antigenic structure, whereas internal epitopes are generally more highly conserved.

OprF is the major porin protein in *P. aeruginosa* that forms channels through the outer mem-

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brane that are large enough to accommodate di- and tri-saccharides [9]. In addition, a second function in both outer membrane and cell structure and stability was discovered, in that OprF-deficient mutants have rounded morphology and grow well only in high osmolarity media [10,11]. Studies utilizing mAbs specific for OprF have demonstrated that this protein is potentially useful as a target for diagnostic and immunotherapeutic intervention [12,13]. Indeed, Gilleland and colleagues [14] have presented data to demonstrate that immunization with OprF protects against subsequent *Pseudomonas aeruginosa* infections.

Previous studies with two mAbs directed against *Pseudomonas aeruginosa* protein OprF demonstrated the conservation of two surface epitopes that were present in all *P. aeruginosa* strains tested [7]. Furthermore, the *oprF* gene cross-hybridized in all strains and demonstrated rather conserved restriction patterns [8]. However, the restricted number of mAbs tested, and the observation of variation in surface antigenic structure in other bacterial pathogens, led us to examine here whether OprF was an unusual example of an outer membrane protein with general conservation of surface epitopes.

Materials and Methods

Strain and media

P. aeruginosa PAO strains utilized included wild type strain H103 (IATS serotype 5; Hancock and Carey, 1979), its *OprF*::Ω mutant H636 [10] and H692, a mutant unable to produce A and B band LPS side chains (a gift from Dr. J. Lam, University of Guelph, Canada). Strain M2 [13] was an IATS serotype 5 strain (i.e., the same serotype as strain H103). Other *Pseudomonadaceae* strains used were described previously [7,16,17]. Growth media included Mueller Hinton Broth (all strains) and proteose peptone No. 2 (PP2) without (H103, M2) or with (H636) the addition of 200 mM NaCl and 500 μg/ml streptomycin. Strain H692 was grown on 1% Bactotryptone, 0.5% yeast extract, 0.5% NaCl.

Immunological techniques

Enzyme-linked immunoabsorbent assays were performed with wells coated with either whole cells, isolated outer membranes or purified OprF, following procedures previously described by Mutharia and Hancock [17]. Cell surface indirect immunofluorescence labelling was carried out on mid-logarithmic or stationary phase cells collected by centrifugation from 1.5 ml of culture. The cells were washed twice with PBS by centrifugation and resuspended in 200 μl of PBS. This suspension was smeared on a glass slide (that in some experiments was precoated with 1 mg/ml poly-L-lysine) and allowed to air dry, then fixed in 100% ethanol. The slides were then processed following the method of Hofstra et al. [18], with minor modifications as described by Mutharia and Hancock [17]. The slides were examined with a Zeiss microscope fitted with a condenser for fluorescence microscopy and containing a halogen lamp and suitable filters for emission of fluorescein isothiocyanate at 525 nm.

Opsonized phagocytosis of *P. aeruginosa* was performed using a modification of the procedures of Battershill et al. [19]. Mouse peritoneal macrophages from 2–4 months old B6D2(f1) mice were incubated in 8-well chamber slides (NUNC, Naperville, IL) overnight and rinsed twice prior to addition of bacteria, thereby eliminating the cytospin step. Antibody concentrations used were standardized by ELISA with purified OprF. Experiments were carried out on 3 different days, the results from each day were tabulated and subjected separately to the Mann-Whitney test to determine a significant difference from the control (cells opsonized with mAb MA1-3).

Colony immunoblotting [7] and Western immunoblotting [17] were carried out as described previously.

DNA sequencing

The *oprF* gene of *P. aeruginosa* strain H103 [11] was sequenced as described previously using oligonucleotide primers [8], except that the protocols of Applied Biosystems Inc. (ABI) were followed for sequencing with an ABI Model 373 automated fluorescence DNA sequenator. The

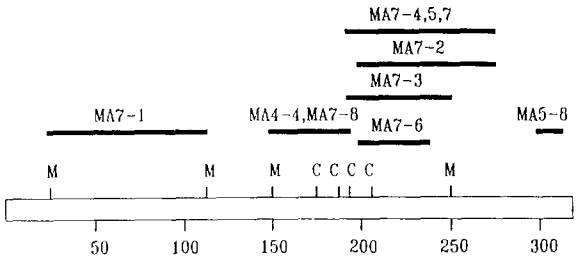

Fig. 1. Linear map of OprF with positions of the methionine (M) and cysteine (C) residues indicated. Above the map are the approximate positions of the monoclonal antibody epitopes based on data from N. Martin, Ph.D. Thesis U.B.C., 1992 and [15]. The numbers refer to amino acid positions.

DNA sequence is deposited in Genbank under the accession number M94078.

Results

Sequence conservation of OprF in Pseudomonas aeruginosa

Previous experiments [8] probing, by Southern hybridization, the *oprF* gene of 59 *P. aeruginosa* clinical and serotyping strains indicated only one restriction endonuclease polymorphism observed in 2 strains which demonstrated an additional *Kpn*1 site in the carboxy terminal half of the *oprF* gene. This unusual pattern was also observed for the *oprF* gene from a serotype 12 isolate that had been sequenced (Genbank accession number P13794) by Duchene et al. [20], whereas the majority of strains, including our laboratory wild type strain H103 and strain M2, lacked this *Kpn*1 site. To determine the extent of variation of *oprF* sequences in *P. aeruginosa*, the cloned gene [11] from strain H103 was sequenced in this study (Genbank accession number M94078). Only 16 nucleotide pair changes were observed relative to the serotype 12 sequence. Nine of these were C → T (at nucleotide positions 69, 175, 225, 486, 624, 650, 789, 954 and 1,029 of the open reading frame), four were T → C (at positions 228, 267, 516, and 837), two were T → G (at positions 150 and 204), and one was G → A (at position 930). All of these changes were silent and thus the two amino acid sequences were identical.

Antigenic conservation of OprF in Pseudomonas aeruginosa

A panel [15] of ten mAbs recognizing diverse epitopes of OprF (Fig. 1) were reacted with a wide variety of *P. aeruginosa* strains using the colony immunoblot procedure [7]. All isolates of *P. aeruginosa*, except for the negative control strain H636 *oprF::Ω*, reacted on colony immunoblots with all 10 mAbs (Table 1), as confirmed in selected cases by Western immunoblot-

Table 1
Monoclonal antibody reactivity of various Pseudomonads of rRNA homology group I

Strains [a]	Reactivity [b]									
	MA4-4	MA5-8	MA7-1	MA7-2	MA7-3	MA7-4	MA7-5	MA7-6	MA7-7	MA7-8
P. aeruginosa (46 isolates)	++	++	++	++	++	++	++	++	++	++
P. syringae ATCC 19310^T	++	−	−	++	−	−	−	++	−	++
P. fluoresccens ATCC 13525^T	−	−	−	−	+	−	−	−	++	−
P.fluorescens OE28.3	− [c]	−	−	++	−	+	−	++	++	−
P. putida ATCC 12633^T	++	−	−	++	+	+	+	+	++	++
P. stutzerii ATCC 17578^T	+	−	−	+	++	−	−	++	++	−
P. aureofaciens ATCC 13985^T	−	−	+	++	−	++	−	+	++	−
P. chloraphis ATCC 9446^T	−	−	+	++	−	++	−	+	++	−
A. vinelandii OP	−	−	−	+	+	−	−	−	+	−

[a] The designation superscript T indicates that this is the type strain of the species.

[b] ++ Reaction on Western immunoblot equivalent to OprF of *P. aeruginosa* strain H692; + reaction of lesser intensity than that for *P. aeruginosa* OprF; − no reaction. All samples were heated at 100°C for 15 min in solubilization reduction mix without 2-mercaptoethanol, prior to SDS-PAGE. Results for the 46 *P. aeruginosa* strains were obtained by colony immunoblot [7] and validated in selected instances by Western immunoblot.

[c] Reactivity was observed with a band of lower apparent molecular mass.

ting and ELISA studies. The *P. aeruginosa* strains tested included the 17 IATS serotype isolates [17], 15 clinical strains from different countries, 15 environmental isolates, and 3 laboratory isolates. These data thus indicated strong conservation of OprF epitopes in *P. aeruginosa*.

Reactivity with other Pseudomonadaceae

Southern blotting studies with the *oprF* gene from *P. aeruginosa* previously indicated that the *oprF* gene was conserved in all *Pseudomonadaceae* of rRNA homology group I [8]. Two additional genes have now been sequenced, the *P. syringae* pv. *syringae* ATCC 19310 OprF porin [8] and the *P. fluorescens* OE283 plant root adhesion protein [21]. Interestingly, although both have extensive amino acid homology with *P. aeruginosa* OprF, the latter lacks the entire cysteine-containing region of OprF, whereas the former contains this region [21]. Consistent with this, *P. syringae* OprF reacted with mAbs MA4-4 and MA7-8, which recognize the cysteine disulphide region [15], whereas *P. fluorescens* OE283 did not (Table 1).

Of the *Pseudomonadaceae* strains examined by Western immunoblot analysis (Table 1), *P. putida* showed the broadest cross-reactivity of any of the strains examined here, reacting with 8 of the 10 mAbs. However, all strains examined reacted with 2 or more mAbs. The weakest cross-reactivity was observed with ATCC 13525, the type strain of *P. fluorescens*, and with *Azotobacter vinelandii*. Three mAbs MA7-2, MA7-6 and MA7-7 represented the most conserved epitopes reacting with 7, 7 and 8 strains, respectively. Conversely, the epitopes for MA5-8, MA7-5 and MA7-1 were poorly conserved demonstrating weak reactivity with 0 to 2 other Pseudomonads.

Surface localization studies. It has been previously demonstrated that surface localized regions of outer membrane proteins demonstrate the greatest variability [3,4,22]. Thus, it was possible that the antigenic conservation described above reflected conservation of epitopes that were not exposed on the surface. Therefore, indirect immunfluorescence studies were performed to determine whether or not the OprF epitopes being studied were surface localized (Table 2). Utilizing strain M2, we observed labelling of the surfaces of cells with all antibodies studied except MA7-2 and the negative control [19] antibody MA1-3. Labelling of strain H103 was consistently weaker and several antibodies failed to label strain H103. This appeared to be due in part to masking of OprF by LPS side chains since strain H692, an LPS-altered, rough derivative of strain H103 was better labelled with all mAbs. Strain H636, an *oprF*::Ω mutant of strain H103, was not labelled by any of the mAbs.

Table 2
Indirect immunofluorescence labelling of intact *P. aeruginosa* by OprF-specific monoclonal antibodies and the control antibody MA1-3

Monoclonal antibody	Immunofluorescence [a]			
	M2	H103	H692	H636
MA4-4	+ +	+ +	+ +	
MA5-8	+ +	–	+ +	–
MA7-1	+ +	–	+	–
MA7-2	–	–	+	–
MA7-3	+ +	+	+	–
MA7-4	+ +	+	+ +	–
MA7-5	+	+	+ +	–
MA7-6	+	–	+	–
MA7-7	+ +	+	+ +	–
MA7-8	+ +	–	+ +	–
MA1-3 [b]	–	–	NT [c]	NT

[a] – No fluorescence or only weak fluorescence observed, + cells uniformly fluorescent, + + cells highly fluorescent.
[b] Negative control antibody specific for a non surface-localized epitope on outer membrane protein OprI.
[c] NT, not tested.

As an alternative method of studying surface binding, each of the mAbs was utilized as an opsonin for phagocytosis of strain M2 by non-elicited mouse peritoneal macrophages. We confirmed previous data [19] which suggested that MA5-8 and MA4-4 were opsonic and that MA5-8 was superior to MA4-4 in opsonizing for phagocytosis (Fig. 2). At an antibody dilution of 10^{-2} MA7-3, MA7-5, MA7-6, MA7-7 and MA7-8 also consistently opsonized *P. aeruginosa* for phagocytosis. MA7-2 was no better than the control antibody MA1-3 whereas MA7-1 and MA7-4 resulted in a significant increase in phagocytosis in 1 out of 3 experiments. When less diluted (10^{-1}) antibody was also utilized for phagocytosis, MA7-1 consistently gave significant phagocytosis, while

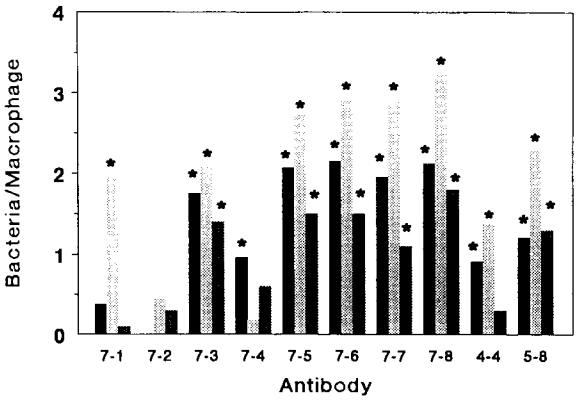

Fig. 2. Enhancement of phagocytosis of *P. aeruginosa* strain M2 by OprF-specific monoclonal antibodies. Results represent the average number of bacteria associated per mouse peritoneal macrophage. Each different shaded bar represents data from an independent experiment with the control number of bacteria per macrophage (obtained using the negative control antibody MA1-3) subtracted. The star indicates samples that were significantly different from the control (by Mann-Whitney test $P < 0.05$).

other antibodies yielded the same types of results as shown in Fig. 2. Overall the data suggested that the epitope for MA7-2 is not exposed (except perhaps in the LPS-altered strain H692). However, all other epitopes were apparently surface exposed.

Discussion

In contrast to several outer membrane proteins found in other bacterial species [2–6], OprF was demonstrated here to contain a number of discrete, highly conserved surface antigenic epitopes. The mAbs utilized in this study recognized epitopes that were largely clustered in the carboxy terminal half of the protein. Nevertheless, a variety of data including their variable reactivity with OprF equivalents from other Pseudomonads (Table 1) and their differential reactivity with peptides [15; N. Martin, Ph.D. Thesis, U.B.C., 1992] indicate that they recognized separate epitopes. The reason for conservation of OprF epitopes was not definitively determined in this study. In other bacteria, it has been suggested that masking of outer membrane protein surface epitopes by LPS limits their reactivity with antibodies specific for these epitopes [23]. This also appeared evident in this study comparing strain H103 with its LPS-altered, rough derivative H692 (Table 2). However, this cannot be the only important factor since immunofluorescence labelling of strain H103 was observed with some mAbs and one of those mAbs MA4-4 [13] protects mice against *Pseudomonas* infections. Furthermore, cystic fibrosis patient isolates, that demonstrate rough LPS, were fully reactive with all 10 mAbs, indicating that these isolates had not undergone extensive *oprF* gene mutations.

Pseudomonas species from rRNA homology group I demonstrated immunologic cross-reactivity with subsets of the 10 OprF-specific mAbs, indicating that OprF has been conserved across species barriers. Three of the *Pseudomonas* species tested have now had their *oprF* genes sequenced. Alignment of the sequences of the OprF proteins from *P. aeruginosa* [20], *P. syringae* [8] and *P. fluorescens* [21] revealed that the carboxy-terminal 121 amino acids are highly conserved. Compared to *P. aeruginosa, P. syringae* OprF has 101 identical amino acids, 12 conservative substitutions and 8 differences while *P. fluorescens* OprF [21] has 1 single amino acid deletion, 13 different residues, 19 conservative substitutions and 87 identical amino acids (providing the cysteine disulphide region is excluded from this analysis). Only two (MA7-2, MA7-6) of the 7 mAbs specific for this region of OprF, crossreacted strongly with all 3 proteins. mAb MA7-2 did not apparently recognize an epitope that is well exposed on the surface of *P. aeruginosa* (Fig. 1). Thus, it seems reasonable to conclude that in these different species, the surface-localized regions, recognized by 5 of the other 6 mAbs with epitopes in the carboxy terminal 121 amino acids, have undergone the greatest antigenic variation. Consistent with this idea, the surface epitopes of porin I from *Neisseria* [24] and FepA from *Enterobacteriaceae* [22] represent the most variable portions of these proteins.

Acknowledgements

Financial support for this project came from the Medical Research Council of Canada. E.R.

and R.W. were supported by studentships from the Canadian Cystic Fibrosis Foundation and Natural Sciences and Engineering Research Council of Canada, respectively.

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