

## Characterization of two surface-localized antigenic sites on porin protein F of *Pseudomonas aeruginosa*

LUCY M. MUTHARIA AND ROBERT E. W. HANCOCK<sup>1</sup>

Department of Microbiology, University of British Columbia, Vancouver, B.C., Canada V6T 1W5

Accepted January 15, 1985

MUTHARIA, L. M., and R. E. W. HANCOCK. 1985. Characterization of two surface-localized antigenic sites on porin protein F of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* **31**: 381–386.

A rapid colony immunoblot screening procedure was used to demonstrate the surface localization of porin protein F on bacterial colonies of *Pseudomonas aeruginosa*. By this method, we demonstrated that protein F was accessible to four different specific monoclonal antibodies in a wide variety of both mucoid and nonmucoid *P. aeruginosa* strains. Controls were performed to demonstrate that, using this procedure, only surface-exposed epitopes bound monoclonal antibodies and that nonspecific binding of monoclonal antibodies either to cells lacking protein F or to mucoid exopolysaccharide did not occur. Monoclonal antibodies MA4-4, MA2-10, and MA4-10, specific for protein F, also interacted with colonies of *Pseudomonas putida* and *Pseudomonas syringae*, whereas the protein F specific monoclonal antibody MA5-8 interacted only with *P. aeruginosa* strains. Using the above-named monoclonal antibodies, we investigated the antigenic structure of protein F. Monoclonal antibodies MA4-4, MA2-10, and MA4-10 bound to 29–31 kilodalton proteolytic fragments produced after papain or trypsin digestion of purified protein F or of protein F in outer membranes or intact cells. Antibody MA5-8 did not interact with proteolytically digested protein F but did interact with two of the six fragments produced after partial cyanogen bromide cleavage of protein F. Antibodies MA4-4, MA2-10, and MA4-10 did not interact with protein F after reduction of its internal disulphide bonds with 2-mercaptoethanol; in contrast, the reactivity of MA5-8 was unaffected. This data suggests that there are at least two distinct highly conserved surface epitopes on porin protein F.

MUTHARIA, L. M., et R. E. W. HANCOCK. 1985. Characterization of two surface-localized antigenic sites on porin protein F of *Pseudomonas aeruginosa*. *Can. J. Microbiol.* **31**: 381–386.

Une technique rapide d'échantillonnage par "immunoblotting" directement à partir de colonies de *Pseudomonas aeruginosa* a servi à démontrer la localisation en surface de la protéine F des porines. Cette méthode a permis de démontrer que la protéine F pouvait réagir avec quatre anticorps monoclonaux différents chez plusieurs souches de *P. aeruginosa* muqueuses ou non-muqueuses. Des études-contrôles ont permis de bien vérifier que seuls les épitopes bien localisés à la surface réagissaient avec les anticorps monoclonaux et qu'il n'y avait pas de réaction non-spécifique entre ces anticorps avec des bactéries dépourvues de la protéine F ou avec l'exopolysaccharide de la couche muqueuse. Les anticorps monoclonaux MA4-4, MA2-10 et MA4-10 spécifiques à la protéine F réagissaient avec les colonies de *Pseudomonas putida* et de *Pseudomonas syringae*, alors que l'anticorps monoclonal MA5-8 spécifique à la protéine F réagissait seulement avec les souches de *P. aeruginosa*. À l'aide des anticorps identifiés précédemment, nous avons entrepris d'étudier la structure antigénique de la protéine F. Ainsi les anticorps MA4-4, MA2-10 et MA4-10 réagissaient avec des fractions protéolytiques de 29 à 31 kilodaltons obtenues par digestion par la trypsine ou la papaine de la protéine F dans la membrane externe ou dans des cellules intactes. L'anticorps MA5-8 ne réagissait pas avec cette protéine F traitée par des enzymes protéolytiques mais réagissait avec deux des six fractions obtenues après un bris partiel de la protéine F par le bromure de cyanogène. Les anticorps MA4-4, MA2-10 et MA4-10 ne réagissaient pas avec la protéine F après réduction des ponts disulfurés internes avec le 2-mercaptoéthanol mais, par contre, la réactivité des anticorps MA5-8 ne changeait pas. Ces résultats laissent croire que sur la protéine F des porines il existe au moins deux épitopes de surface distincts régulièrement exprimés.

[Traduit par le journal]

### Introduction

The protein and lipopolysaccharide (LPS) molecules of the outer membranes of gram-negative bacteria present a complex mosaic of antigens on the bacterial cell surface. The structure, function, and immunological properties of the polysaccharide "O" antigen, the rough core region, and the lipid A region of LPS have been the subject of extensive investigations. In contrast, studies on outer membrane proteins have not progressed at the same rate.

The outer membrane of *Pseudomonas aeruginosa* contains up to nine different polypeptides expressed in very high copy numbers and hence called major outer membrane proteins (Hancock and Carey 1979). One of these proteins, protein F, forms water-filled channels through the hydrophobic core of the outer membrane (Hancock *et al.* 1979), and thus belongs to the class of proteins termed porins (Nakae 1976). Protein F is tightly but noncovalently associated with the peptidoglycan (Hancock *et al.* 1982) and is surface exposed as demonstrated by indirect immunofluorescence studies using specific mono-

clonal antibodies to protein F (Mutharia and Hancock 1983). Using both polyclonal and monoclonal antibodies we have previously shown, by ELISA and Western immunoblotting, the antigenic conservation of protein F (Mutharia *et al.* 1982; Hancock *et al.* 1983) amongst the different serotypes and a variety of clinical isolates of *P. aeruginosa*.

The present investigation looks at the application of protein F specific monoclonal antibodies to the rapid screening of bacterial colonies for cell-surface antigenic sites (epitopes) recognized by these antibodies. Surface-localized epitopes were studied in a wide range of gram-negative bacteria and the taxonomic importance of the results is discussed. We have also reported studies on the characterization, using these monoclonal antibodies, of the antigenic domains of the porin protein F.

### Materials and methods

#### Bacterial strains

The 17 *P. aeruginosa* strains representing the serotypes of the International Antigen Typing Scheme were described by Mutharia *et al.* (1982). The *P. aeruginosa* clinical isolates from cystic fibrosis

<sup>1</sup>Author to whom all correspondence should be addressed.

TABLE 1. Use of the colony blot procedure to detect antigenic cross-reactivity of various bacterial strains with monoclonal antibodies specific for surface-located epitopes on *P. aeruginosa* protein F

Bacterial strains	Binding of monoclonal antibodies		
	MA2-10	MA4-4	MA5-8
<i>P. aeruginosa</i> strains			
H103	+	+	+
H283 (protein F deficient)	-	-	-
Laboratory isolates (6 strains) <sup>a</sup>	+	+	+
Serotype-specific strains (17 strains) <sup>b</sup>	+	+	+
Clinical isolates (35 strains) <sup>c</sup>	+	+	+
<i>P. putida</i> strains (2 strains) <sup>d</sup>			
<i>P. syringae</i> strain ATCC 19310	+	+	-
Other <i>Pseudomonadaceae</i> strains (10 strains) <sup>e</sup>	-	-	-
Other gram-negative bacteria (9 strains) <sup>f</sup>	-	-	-

<sup>a</sup>Bacterial strains used were *P. aeruginosa* strains Z61, K799, AK1012, AK1282 and H223 as described by Hancock *et al.* (1982); and *P. aeruginosa* ATCC 19305.

<sup>b</sup>The serotype strains of the International Antigen Typing Scheme were as described by Hancock *et al.* (1982).

<sup>c</sup>Fifteen *P. aeruginosa* strains (CF832, CF1452, CF2314, CF4522, CF2314, CF4522, CF3660-1, CF6094, L; CF4349, CF1278, CF221, CF284, CF3790, and CF9490) were as described by Hancock *et al.* (1983) and another 10 mucoid *P. aeruginosa* isolates from cystic fibrosis patients (C1m; C21m, C20m; C6m; C81m; C47m; C46m; C4m; C91m, and C96m) and their spontaneous nonmucoid variants, were obtained from D. P. Speert, Children's Hospital, Vancouver, B.C.

<sup>d</sup>*Pseudomonas putida* strains ATCC 4359 and ATCC 12633.

<sup>e</sup>*Pseudomonas chlororaphis* ATCC 9446; *P. maltophilia* ATCC 13637; *P. pseudomallei* ATCC 2343; *P. solanacearum* ATCC 11696; *P. stutzeri* ATCC 17588; *P. syringae* ATCC 19310; *P. aureofaciens* ATCC 13985; *P. cepacia*; *P. fluorescens* ATCC 949 and ATCC 13525.

<sup>f</sup>*Salmonella typhimurium* LT2 SGSC205 and SGSC227; *Vibrio anguillarum* HT7602; *Aeromonas hydrophila* ET2; *Aeromonas salmonicida* NCMB 2020; *Escherichia coli* CGSC6041, CGSC6044 and PC 0479; *Edwardsiella tarda* E79054.

patients were described by Hancock *et al.* (1983). Other *P. aeruginosa* strains were as previously described (Hancock *et al.* 1983; Mutharia *et al.* 1982; Mutharia and Hancock 1983). All other strains were described by Nakajima *et al.* (1982).

#### Antigen preparation

Outer membranes from all strains were isolated by the one-step sucrose gradient method of Hancock and Carey (1979). Proteins F and H2 were purified from *P. aeruginosa* strain H103 by L. Chan (Microbiology, University of British Columbia), using the method of Hancock *et al.* (1979). Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis was as described by Hancock and Carey (1979). All outer membrane samples were solubilized at 88°C for 10 min before electrophoresis. Whole-cell proteins were solubilized by heating in 2% SDS, 20 mM Tris-HCl (pH 7.4) buffer at 88°C for 10 min, and then prepared for electrophoresis as described previously (Hancock and Carey 1979).

#### Western electrophoretic blot transfer

Transfer of proteins from SDS - polyacrylamide gel electrophoretograms and subsequent immunostaining followed the method described by Mutharia and Hancock (1983).

#### Colony blotting

Colony blotting was performed by a modification of the method of Henning *et al.* (1979). Bacterial colonies were transferred from agar plates onto prewashed nitrocellulose filters by contact. After incubation of the filters at 30°C for 30 min, the colony blots were successively incubated with Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4; 2 mM MgCl<sub>2</sub>, 1 mM NaCl) containing 3% (w/v) gelatin, for 45 min at 37°C, then for 2 h with the test antibody diluted in TBS buffer containing 1% gelatin. The blots were subsequently washed three times by incubation for 15 min in TBS containing 0.1% gelatin, and subsequently incubated for 2 h at 37°C with a goat anti-mouse immunoglobulin coupled to horse-radish peroxidase (Flow Labs Inc., McLean, VA). After washing as above, the blots were developed

using a histochemical substrate for peroxidase (5 mg of 4-chloro-1-naphthol dissolved in 1.67 mL of ice-cold absolute methanol and mixed at 23°C with 10 mL of TBS containing 5 µL of 30% (v/v) H<sub>2</sub>O<sub>2</sub>).

#### Chemical cleavage of protein F

Chemical cleavage of protein F by cyanogen bromide followed the method of Garten and Henning (1974). Briefly, 60 µg of lyophilized purified protein F were dissolved in 0.8 mL of 98% (v/v) formic acid containing 1 M cyanogen bromide and 0.6 N HCl. The volume was made up to 1 mL. The sample was incubated at 37°C in a foil-covered tightly capped vial for 18 h, and then diluted and lyophilized. The residue (containing cyanogen bromide peptides) was redissolved in deionized water and re-lyophilized. The lyophilized peptides were then redissolved in the original volume of 10% SDS, 10 mM Tris-HCl (pH 7.4) buffer. In some cases, protein F was denatured in 80% acetic acid for 1 h prior to cyanogen bromide treatment.

#### Enzymatic digestion of protein F

Fifty microlitres of purified protein F (at 1 mg/mL) or of protein F in outer membranes and whole cells (at 1 mg total in protein) was enzymatically digested with the following enzymes. (i) *Staphylococcus aureus* V8 protease (Sigma Chemical Co., St. Louis, Mo.) in 20 mM Tris-HCl, pH 7.4 containing 35 mM MgCl<sub>2</sub>·6H<sub>2</sub>O. The enzyme:protein ratio was 1:100. (ii) Papain (papainase Type 4, Sigma) in 2 mM EDTA - 20 mM Tris-HCl, pH 6.0, at 50 µg enzyme/mg protein. (iii) L-1-Tosylamide-2-phenylethylchloromethyl ketone (TPCK) - trypsin (Sigma) in 10 mM Tris-HCl, pH 8.0, at 0.1 mg enzyme/mg protein. The digestions were performed at 37°C for 60 min.

#### Monoclonal antibodies

Monoclonal antibodies were grown in ascites and purified by ammonium sulphate precipitation. The following monoclonal antibodies described previously (Mutharia and Hancock 1983; Mutharia *et al.* 1982) were used: MA5-8, MA4-4, MA2-10, and MA4-10, specific

for protein F; monoclonal antibody MA1-8, specific for *P. aeruginosa* serotype 5 LPS 'O' antigen; and MA1-6, specific for lipoprotein H2.

## Results

### Colony blot screening

Monoclonal antibodies MA4-4, MA2-10, MA4-10, and MA5-8 were interacted by the colony blotting procedure with the type strains from all 17 serotypes (International Antigen Typing Scheme) of *P. aeruginosa*, a variety of laboratory strains as well as mucoid *P. aeruginosa* isolates, obtained from patients with cystic fibrosis, and their spontaneous nonmucoid revertants (Table 1). Although we observed differences in the intensity of the blue colour developed with positive (antibody-binding) colonies, all *P. aeruginosa* strains tested interacted with the monoclonal antibodies (Fig. 1). In contrast, a *P. aeruginosa* strain H283 that lacks protein F (Nicas and Hancock 1983) and CF283, a non-*P. aeruginosa* gram-negative isolate from a cystic fibrosis patient, did not bind any of the protein F specific monoclonal antibodies.

Mucoid colonies of *P. aeruginosa* and their nonmucoid revertants did not show any differences in their interaction with the antibodies by the colony blot procedure (Fig. 1), despite the fact that the mucoid material (alginate exopolysaccharide) appeared to have been transferred to the nitrocellulose blot together with the bacterial cells. The mucoid material did not apparently mask the accessibility of the protein to the antibody nor did it absorb antibodies nonspecifically to the surface of the colony. The lack of nonspecific antibody binding to mucoid material was demonstrated by the lack of interaction of monoclonal antibody MA1-6 (specific for protein H2), with colonies of a mucoid derivative of strain CF46, whose outer membranes were previously (L. Mutharia, unpublished data) demonstrated to be deficient in protein H2 by both SDS - polyacrylamide gel electrophoresis and Western immunoblot analysis.

As a further control, we demonstrated, using colonies of the serotype strains of *P. aeruginosa* and the cystic fibrosis isolates, that monoclonal antibody MA1-8, specific for serotype 5 LPS O-antigen, showed positive binding to serotypes 5, 17, and CFC1 mucoid and nonmucoid, but no interaction with any of the other colonies (Table 1). Thus, the colony blot results reflected the specificity of antibody MA1-8 as shown previously by enzyme-linked immunosorbent assay (ELISA) (Hancock *et al.* 1982). In addition, monoclonal antibody MA1-6, which is specific for outer membrane lipoprotein H2 (Hancock *et al.* 1982), interacted only with colonies of rough LPS altered mutants of *P. aeruginosa* and not with smooth strains (L. M. Mutharia and R. E. W. Hancock, to be published). However, monoclonal antibody MA1-6 interacted on Western blots (L. M. Mutharia and R. E. W. Hancock, to be published) and ELISA (Hancock *et al.* 1982) with outer membranes from both the smooth and the rough strains. In addition the antibody also interacted with the smooth strains when MgCl<sub>2</sub> was omitted and 0.1% (w/v) SDS and 0.1 M NaCl were added to the buffers used for the colony blotting. This confirmed our previous data from indirect immunofluorescence studies which suggested that protein H2 is not expressed on the surface of wild-type (smooth) *P. aeruginosa* strains (Mutharia and Hancock 1983). Furthermore, it indicated that the colony blotting procedure was specific for surface-localized epitopes (unless the colonies were subjected to the above procedures).

### Interaction of protein F specific monoclonal antibodies with other bacteria

The interaction of protein F specific monoclonal antibodies

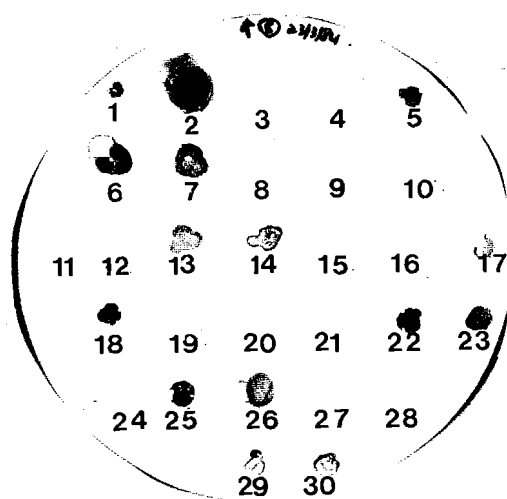


FIG. 1. A colony immunoblot showing interaction of *P. aeruginosa* protein F specific monoclonal antibody MA4-10 with the following strains: 1, *P. aeruginosa* strain P1 nonmucoid; 2, *P. aeruginosa* strain P1 mucoid; 3 and 4, *P. fluorescens* ATCC 949 and 13525 (type strain), respectively; 5, *P. aeruginosa* PA01 strain H103; 6, *P. aeruginosa* strain C1 mucoid; 7, *P. aeruginosa* strain C1 nonmucoid; 8, 9, and 10, *Escherichia coli* strains CGSC6041, CGSC6044, and PC0479; 11, *P. pseudomallei* ATCC23343; 12, *P. solanacearum* ATCC 11696; 13 and 14, *P. putida* ATCC 4359 and 12633 (type strain), respectively; 15 and 16, *Salmonella typhimurium* LT2 strains SGSC206 and SGSC227, respectively; 17, *P. aeruginosa* ATCC 9721; 18, *P. aeruginosa* PA01 strain H103; 19, *Aeromonas salmonicida* NCMB 2020; 20, *Aeromonas hydrophila* ET2; 21, *P. chlororaphis* ATCC 9446; 22, *P. aeruginosa* (type strain) ATCC 19305; 23, *P. aeruginosa* strain Z61; 24, *P. aureofaciens* ATCC 13985; 25, *P. syringae* ATCC 19310; 26, *P. aeruginosa* CF4349; 27, *P. maltophilia* ATCC 13639; 28, *P. stutzeri* ATCC 17588; 29 and 30, *P. aeruginosa* strains AK1012 and AK1282, respectively.

with other *Pseudomonas* strains and various gram-negative bacteria was studied by both the colony blot procedure described above (Table 1) and by Western immunoblotting using, as antigens, either cell envelopes or whole-cell lysates transferred to nitrocellulose. Monoclonal antibody MA5-8 was highly specific for *P. aeruginosa* strains, while MA4-4, MA2-10, and MA4-10 interacted with both strains of *P. putida* and the single *P. syringae* strain tested (Table 1). None of these monoclonal antibodies interacted with whole cells or cell envelopes of *P. fluorescens*, *P. anguilliseptica*, *P. chlororaphis*, *P. aureofaciens*, *P. stutzeri*, or *Azotobacter vinelandii*, all of which have been previously demonstrated to be related to *P. aeruginosa* in rRNA homology experiments (DeVos and DeLey 1983). There was no interaction with any of the strains from the *Enterobacteriaceae*, *Vibrionaceae*, or other *Pseudomonads*, e.g., *P. cepacia*.

### Antigenic domains of protein F

The high specificity of monoclonal antibodies has been used to study the structure and antigenic domains of proteins (Kenimer *et al.* 1983; Virji *et al.* 1983). We used the protein F specific monoclonal antibodies MA5-8, MA4-4, MA2-10, and MA4-10 in an attempt to define some of the antigenic domains (epitopes) of this protein.

The apparent molecular weight or migration of protein F of *P. aeruginosa* on SDS - polyacrylamide gels is markedly influenced by the solubilization conditions (Hancock and Carey

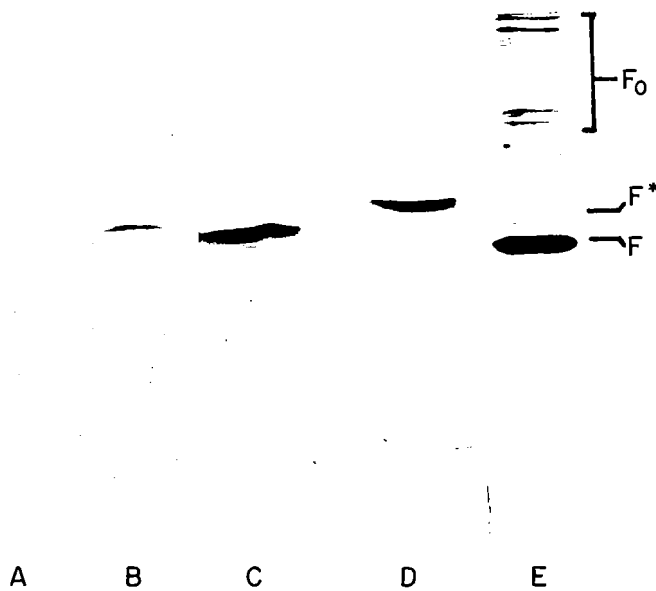


FIG. 2. Western immunoblot of purified protein F. Effect of 2-mercaptoethanol. Purified protein F was separated on SDS - polyacrylamide gel electrophoretograms after solubilization (lanes A and D) with or (lanes B, C, and E) without 2-mercaptoethanol. After electrophoretic transfer to nitrocellulose, the blots were interacted with MA4-4 (lanes A and B) or MA5-8 (lanes C, D, and E) and then the position of the bound monoclonal antibodies revealed as described previously (Mutharia and Hancock 1983). Lane E received five times more protein F (5  $\mu$ g) than lanes A-D, to demonstrate the putative oligomer bands not visible in lanes A-D. The banding positions are marked F (for the 2-mercaptoethanol untreated protein F), F\* (for the 2-mercaptoethanol reduced protein F), and F<sub>0</sub> (for the putative protein F oligomers).

1979). When solubilized in the presence of 2-mercaptoethanol, protein F migrates as a 41 000 dalton protein on a 14% polyacrylamide gel. The nonreduced protein has an apparent molecular weight of 37 000 (Hancock and Carey 1979). The reason for this is almost certainly the presence of one or two intrachain disulphide bonds. Thus, when these disulphide bonds are not reduced, the protein runs in a more compact configuration with a higher relative mobility. Monoclonal antibodies MA4-4, MA2-10, and MA4-10 interacted only with the nonreduced form of protein F, while MA5-8 interacted with both the 2-mercaptoethanol reduced and nonreduced forms (Fig. 2).

Monoclonal antibody MA5-8 was also unique in that it interacted with higher molecular weight (putative oligomeric) forms of protein F (Fig. 2). These forms of protein F were not observed when outer membranes of the protein F deficient mutant H283 were used in Western immunoblot studies, suggesting that they did not represent artefacts owing to cross-reaction of monoclonal antibody MA5-8 with other proteins. These apparent oligomeric associations of protein F were present in very low concentrations in outer membranes but were enriched during purification of protein F. This data provided the first evidence for the possible existence of SDS-stable oligomeric forms of protein F.

#### *Interaction of monoclonal antibodies with cyanogen bromide and proteolytic peptide fragments of protein F*

Peptide fragments of protein F were derived by both chemical cleavage with cyanogen bromide and by enzymatic digestion with trypsin, papain, or V8 protease. Cyanogen bro-

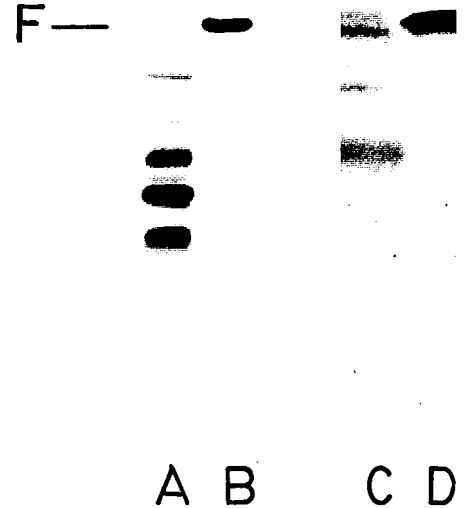


FIG. 3. SDS - polyacrylamide gel electrophoretogram and Western immunoblot of purified protein F before and after degradation with cyanogen bromide. Lanes A and C, cyanogen bromide peptides of protein F; lanes B and D, purified protein F. Protein F and its peptides were transferred from an SDS - polyacrylamide gel (lanes A and B) to nitrocellulose (lanes C and D) and interacted with monoclonal antibody MA5-8 on the nitrocellulose. The two cyanogen bromide peptides of protein F which interact with antibody MA5-8 had molecular weights of 28 000 and 23 000. In the cyanogen bromide cleavage pattern in lane C, the native protein F formed a doublet owing to partial heat modification of the undegraded protein F (Hancock and Carey 1979). This small residue of undegraded protein F preferentially bound the monoclonal antibody, since it was not visible in the gel pattern in lane A. The molecular weight of the native protein F band (unreduced by 2-mercaptoethanol) in lane B was 36 000. The molecular weights of the bands visible in lane A (as determined by reference to molecular weight standards) were 28 000, 25 500 (minor band), 23 000, 21 500 (minor band), 20 000, and 17 000, respectively, in descending order.

mid treatment of native or denatured protein F yielded six fragments, two of which had altered mobility in the presence of 2-mercaptoethanol suggesting internal disulphide bonds (Fig. 3). The total molecular weights of these peptides, however, was approximately three times that of monomeric protein F. The discrepancy in molecular weight is probably due to incomplete cleavage. This has been shown to occur, e.g., owing to incomplete cleavage of Met-Ser or Met-Thr sequences which can form homoserine and are therefore not cleaved, leading to the production of overlapping cyanogen bromide fragments of total molecular weights greater than the native protein (Garten and Henning 1974). This incomplete cleavage was also observed with denatured protein, and under more rigorous digestion conditions. Only monoclonal antibody MA5-8 interacted with any of the fragments. Specifically, it interacted with the two 2-mercaptoethanol modifiable fragments of protein F in both the reduced and nonreduced forms (Fig. 3). The inability to generate smaller antibody reactive

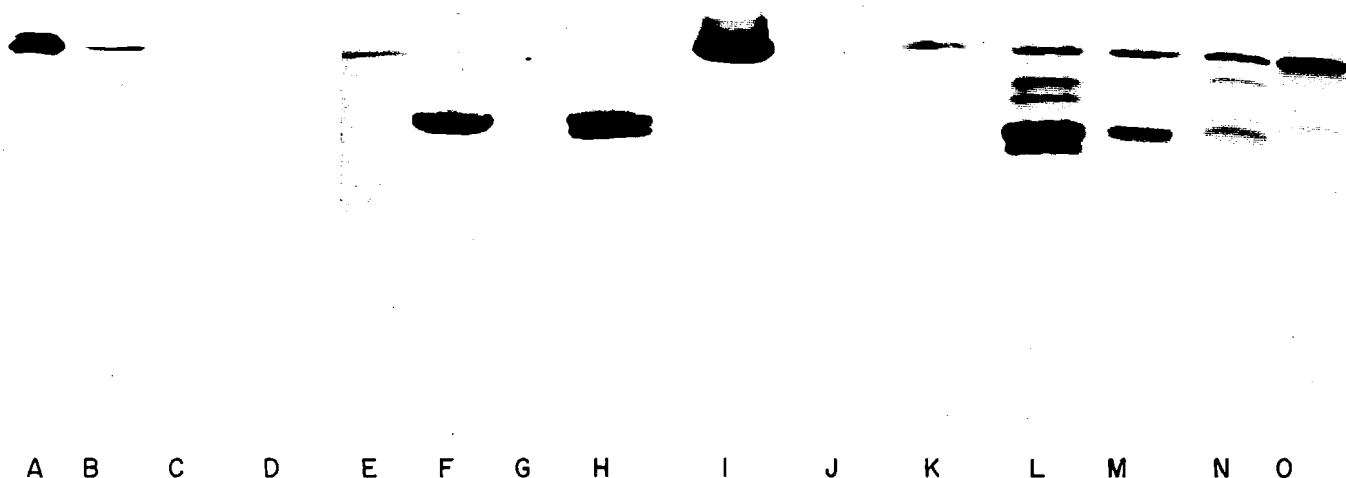


FIG. 4. Western immunoblot of native and proteolytically treated protein F from a purified protein F sample (lanes A–H), outer membranes (lanes I–M), and whole cells lanes (lanes N and O). The proteolytic enzymes and the monoclonal antibodies used to reveal the positions of the native protein F and its proteolytic fragments were as follows: lane A, no treatment, antibody MA5-8; lane B, trypsin, MA5-8; lane C, *S. aureus* V8 protease, MA5-8; lane D, papain, MA5-8; lane E, no treatment, MA4-10; lane F, trypsin, MA4-10; lane G, V8 protease, MA4-10; lane H, papain, MA4-10; lane I, no treatment, MA5-8; lane J, papain, MA5-8; lane K, trypsin, MA5-8; lane L, papain, MA4-10; lane M, trypsin, MA4-10; lane N, papain, MA4-10; lane O, trypsin, MA4-10.

peptides under harsher conditions (pretreatment with 85% glacial acetic acid) probably reflects the requirement for stabilization of the antigenic epitope by maintenance of the SDS-resistant,  $\beta$ -sheet conformation of protein F (Mizuno and Kageyama 1979), thus limiting the identification of the antigenic domains.

Enzymatic digestion of protein F with *S. aureus* V8 protease for very short times (<1 min) yielded a series of peptides of molecular weights below 12 000, none of which interacted with any of the antibodies. When purified protein F was treated with papain, it rapidly broke down to two peptide fragments with molecular weights of 29 000 and 31 500. Both of these peptides were 2-mercaptoethanol modifiable and reacted with monoclonal antibodies MA2-10, MA4-4, and MA4-10 (in the unreduced form only) but not with MA5-8 (Fig. 4). These two fragments were remarkably resistant to further hydrolysis. Protein F in outer membranes (Fig. 4) and intact cells was also susceptible to papain, although fivefold higher amounts of enzyme were required and digestion did not proceed to completion as readily (especially with whole cells). This may be due to partial masking of the papain-susceptible sites of protein F by LPS. In both outer membranes and intact cells, peptide bands of intermediate molecular weight between the undegraded protein F (37 000) and the above two peptide fragments (29 000 and 31 500) were observed, including bands of 36 000, 35 000, 34 000, 33 000, and 32 500. However, owing to the tendency of protein F to run in a variety of positions on SDS polyacrylamide gels (Hancock and Carey 1979), we are unable to say with certainty that these bands represent intermediates.

Digestion of protein F with trypsin gave similar results. When purified protein F was used, a single 2-mercaptoethanol modifiable peptide fragment of 31 000 formed. This fragment interacted with monoclonal antibodies MA2-10, MA4-4, and MA4-10 (in the unreduced form) but not with MA5-8 (Fig. 4).

Again, protein F in outer membranes and whole cells was more resistant to trypsin, requiring 10 times as much enzyme, and resulting in the appearance of intermediate molecular weight forms of 34 500, 33 500, and 32 500, all of which interacted with MA2-10, MA4-4, and MA4-10. The 31 000 fragment which appeared in purified protein F, outer membrane and intact cell preparations after treatment with trypsin was resistant to further proteolysis. Preliminary results with the protein F equivalent in *P. putida* outer membrane demonstrated that this 39 000 protein broke down to a 31 000, 2-mercaptoethanol modifiable fragment which interacted with monoclonal antibody MA4-10 (data not shown).

### Discussion

The colony immunoblot procedure provided a good rapid screening assay for surface-localized epitopes on cells in bacterial colonies. Protein F was demonstrated to be surface accessible in colonies of both mucoid and nonmucoid *P. aeruginosa* isolates. These results confirmed and extended our observations regarding protein F surface accessibility as assessed by indirect immunofluorescence using monoclonal antibodies (Mutharia and Hancock 1983). The results from the colony blot procedure described here demonstrate that protein F has at least two separate cell-surface antigenic epitopes (recognized by MA5-8 and MA4-4, respectively) that are conserved amongst all *P. aeruginosa* strains.

The epitope recognized by antibodies MA4-4, MA4-10, and MA2-10 is also expressed in *P. putida* and *P. syringae* strains. *Pseudomonas putida* and *P. syringae* together with *P. aeruginosa* have been placed by rRNA homology (DeVos and DeLey 1982) in a closely related cluster as group 1 *Pseudomonadaceae*. Presumably, the lack of interaction of the monoclonal antibodies with other members of that homology group demonstrated that some antigenic drift has occurred with porin protein F. Nevertheless, protein F is far better conserved than

the porin proteins of *Neisseria gonorrhoeae* and *Haemophilus influenzae* (Barenkamp *et al.* 1981) which show antigenic variation within a single species.

The protein F specific monoclonal antibodies fall into two classes on the basis of antigenic cross-reactions. One containing a single monoclonal antibody, MA5-8, which interacts only with *P. aeruginosa* porins, and one containing three monoclonal antibodies, MA4-4, MA2-10, and MA4-10, which cross-react with a protein from *P. putida* and *P. syringae* of similar molecular weight to *P. aeruginosa* protein F. The differences in the antigenic domains recognized by these two classes of antibodies is further demonstrated by the binding of these antibodies to peptide fragments of protein F and to the native protein under different conditions.

Monoclonal antibody, MA5-8, unlike the other antibodies interacted with oligomeric forms of protein F, as well as the 2-mercaptoethanol reduced and nonreduced protein. This antibody also interacted with two partial cyanogen bromide fragments of the protein, although the antigenic site recognized by the antibody was destroyed by either trypsin or papain. In contrast, monoclonal antibodies MA2-10, MA4-4, and MA4-10 interacted with the nonreduced porin, and with the trypsin- and papain-derived fragments of protein F in the nonreduced form. The epitope or epitopes recognized were destroyed by 2-mercaptoethanol as well as by cleavage of the protein with cyanogen bromide.

This data suggests that protein F has at least two surface-located antigenic epitopes (domains) recognized by different antibodies. Both of these domains probably are conformational in that they require some tertiary structure which is maintained in SDS (Mizuno and Kageyama 1979) but destroyed upon rigorous proteolytic or cyanogen bromide degradation. The complex  $\beta$ -structure of porin proteins will require extensive studies with a larger library of monoclonal antibodies before reasonable speculations on the structure of protein F can be made.

#### Acknowledgement

Financial assistance from the Medical Research Council of Canada is gratefully acknowledged.

- BARENKAMP, S. J., R. S. MUNSON, JR., and D. M. GRANOFF. 1981. Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profile. *J. Infect. Dis.* **143**: 668-676.
- DEVOS, P., and J. DELEY. 1983. Intra- and intergenic similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *Int. J. Syst. Bacteriol.* **33**: 487-509.

- GARTEN, W., and U. HENNING. 1974. Cell envelope and shape of *Escherichia coli* K-12: isolation and preliminary characterization of the major ghost-membrane proteins. *Eur. J. Biochem.* **47**: 343-352.
- HANCOCK, R. E. W., and A. M. CAREY. 1979. Outer membrane of *Pseudomonas aeruginosa*: heat and 2-mercaptoethanol-modifiable proteins. *J. Bacteriol.* **140**: 902-910.
- HANCOCK, R. E. W., G. M. DECAD, and H. NIKAIKO. 1979. Identification of the protein producing transmembrane diffusion pores in the outer membrane of *Pseudomonas aeruginosa* PA01. *Biochem. Biophys. Acta*, **554**: 323-331.
- HANCOCK, R. E. W., L. M. MUTHARIA, L. CHAN, R. P. DARVEAU, D. P. SPEERT, and G. B. PIER. 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum sensitive, non-typable strains deficient in lipopolysaccharide O-side chains. *Infect. Immun.* **42**: 170-177.
- HANCOCK, R. E. W., A. A. WIECZOREK, L. M. MUTHARIA, and K. POOLE. 1982. Monoclonal antibodies against *Pseudomonas aeruginosa* outer membrane antigens: isolation and characterization. *Infect. Immun.* **37**: 160-171.
- HENNING, U., H. SCHWERZ, and R. CHEN. 1979. Radio-immunological screening method for specific membrane proteins. *Anal. Biochem.* **97**: 153-157.
- KENIMER, J. G., W. H. HABIG, and M. C. HARDEGOLL. 1983. Monoclonal antibodies as probes of tetanus, toxin structure and function. *Infect. Immun.* **42**: 942-948.
- MIZUNO, T., and M. KAGEYAMA. 1979. Isolation and characterization of major outer membrane proteins of *Pseudomonas aeruginosa* strain PAO with special reference to peptidoglycan associated proteins. *J. Biochem. (Tokyo)*, **86**: 979-989.
- MUTHARIA, L. M., and R. E. W. HANCOCK. 1983. Surface localization of *Pseudomonas aeruginosa* outer membrane porin protein F by using monoclonal antibodies. *Infect. Immun.* **42**: 1027-1033.
- MUTHARIA, L. M., T. I. NICAS, and R. E. W. HANCOCK. 1982. Outer membrane proteins of *Pseudomonas aeruginosa* serotype strains. *J. Infect. Dis.* **146**: 770-779.
- NAKAE, T. 1976. Identification of outer membrane protein of *Escherichia coli* that produces transmembrane channels in reconstituted vesicle membranes. *Biochem. Biophys. Res. Commun.* **71**: 877-884.
- NAKAJIMA, K., K. MUROGA, and R. E. W. HANCOCK. 1983. Comparison of fatty acid, protein and serological properties distinguishing outer membranes of *Pseudomonas anguilliseptica* strains from those of fish pathogens and other *Pseudomonads*. *Int. J. Syst. Bacteriol.* **33**: 1-8.
- NICAS, T. I., and R. E. W. HANCOCK. 1983. *Pseudomonas aeruginosa* outer membrane permeability: isolation of a porin protein F-deficient mutant. *J. Bacteriol.* **153**: 281-285.
- VIRJI, M., J. E. HECKLES, and P. J. WATT. 1983. Monoclonal antibody to gonococcal pili: studies on antigenic determinants on pili from variants of strain P9. *J. Gen. Microbiol.* **129**: 1965-1973.