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Outer Membrane Protein Heterogeneity within *Pseudomonas fluorescens* and *P. putida* and Use of an OprF Antibody as a Probe for rRNA Homology Group I Pseudomonads

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The electrophoretic patterns of outer membrane proteins of strains representing the biovars of *Pseudomonas fluorescens* and *Pseudomonas putida* were analyzed by gel electrophoresis. The outer membrane protein profiles were variable, and they were not useful for assigning strains to a specific biovar. However, three or four predominant outer membrane proteins migrating at 42 to 46 kDa, 33 to 38 kDa, and 20 to 22 kDa were conserved among the strains. They could be tentatively identified as OprE (44 kDa), OprF (38 kDa), OprH (21 kDa), and OprL (20.5 kDa), which are known proteins from *Pseudomonas aeruginosa*. A 37-kDa OprF-like protein was purified from *P. fluorescens* DF57 and used to raise a polyclonal antibody. In Western blot (immunoblot) analysis, this antibody reacted with OprF proteins from members of *Pseudomonas* rRNA homology group I but not with proteins from nonpseudomonads. The heterogeneity in M_r of OprF was greater among *P. fluorescens* strains than among *P. putida* strains. Immunofluorescence microscopy of intact cells demonstrated that the antibody recognized epitopes that were accessible only after unmasking by EDTA treatment. The antibody was used in a colony blotting assay to determine the percentage of rRNA homology group I pseudomonads among bacteria from the rhizosphere of barley. The bacteria were isolated on 10% tryptic soy agar, King's B agar, and the pseudomonad-specific medium Gould S1 agar. The estimate of OprF-containing CFU in rhizosphere soil obtained by colony blotting on 10% tryptic soy agar was about 2 and 14 times higher than the values obtained from King's agar and Gould S1 agar, respectively, indicating that not all fluorescent pseudomonads are scored on more specific media. The colonies reacting with the OprF antibody were verified as being rRNA homology group I pseudomonads by using the API 20NE system.

Pseudomonas rRNA homology group I includes several species of environmental or clinical interest. The opportunistic pathogen *Pseudomonas aeruginosa* is a very homogeneous species and is probably the best-studied fluorescent pseudomonad. *Pseudomonas stutzeri*, on the other hand, is a very heterogeneous species (29), and the same is true for the common saprophytes *Pseudomonas fluorescens* and *Pseudomonas putida*, which are both divided into several biovars (2, 26). A firm distinction between *P. fluorescens* and *P. putida* has been difficult to establish, as some *P. fluorescens* biovars seem to be more closely related to *P. putida* biovar B than to other *P. fluorescens* biovars (2, 4, 18).

Immunochemical techniques are useful for the detection of bacteria in the environment because of the high specificity and sensitivity of these methods. The outer membranes of gram-negative bacteria consist of two classes of macromolecules, lipopolysaccharides (LPS) and proteins, which are accessible for recognition by antibodies. *P. aeruginosa* outer membranes contain a limited number of proteins present in very high numbers (13). Hancock and coworkers (11–13) have developed a set of guidelines for gel electrophoretic analysis and nomenclature of the following proteins: OprD1 (46 kDa), OprD2 (45.5 kDa), OprE (44 kDa), OprF (38 kDa), OprG (25 kDa), OprH (21 kDa), and OprL (20.5 kDa).

It has been demonstrated that the major outer membrane proteins are strongly conserved among different serotypes of *P. aeruginosa* (12, 24). Hence, Western blotting (immunoblotting) with monoclonal antibodies has revealed that OprL proteins from all *P. aeruginosa* serotypes possess common antigenic determinants, whereas OprE proteins from 16 of the 17 serotypes are apparently closely related (24). OprF, the major porin of *P. aeruginosa*, with 10^5 copies per cell, is also conserved among all serotypes (24). This molecule is constitutively expressed (13) and contains surface-exposed epitopes (16, 20, 28). Hence, it constitutes a target for antibodies recognizing *P. aeruginosa* at the species level (20, 22, 23). The *oprF* gene also seems to be conserved within the rRNA homology group I pseudomonads (31), and an OprF-like protein has recently been suggested to be involved in adhesion between a *P. fluorescens* strain and plant roots (5–7).

In the present study, we have determined the heterogeneity of major outer membrane proteins within a strain collection representing all biovars of *P. fluorescens* and *P. putida*. An OprF-like protein was conserved among the strains, and antibodies were raised against OprF from a selected *P. fluorescens* strain. Western blot analysis demonstrated that the antibody was specific to rRNA homology group I pseudomonads. After unmasking with EDTA, OprF was accessible to antibodies in whole-cell assays, and the antibody was used as a probe for detection of rRNA homology group I pseudomonads from rhizosphere soil.

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TABLE 1. Fluorescent *Pseudomonas* sp. isolates

Isolate	Species and biotype[a]	Origin
ON24	*P. fluorescens* I	Soil
MM5	*P. fluorescens* I	Barley rhizosphere
DF8	*P. fluorescens* II	Cucumber rhizosphere
MM6	*P. fluorescens* II	Barley rhizosphere
DF57	*P. fluorescens* III	Cucumber rhizosphere
DF61	*P. fluorescens* III	Cucumber rhizosphere
DF18	*P. fluorescens* IV	Cucumber rhizosphere
DF23	*P. fluorescens* IV	Cucumber rhizosphere
ON13	*P. fluorescens* V	Lake sediment
ON25	*P. fluorescens* V	Lake water
DF17	*P. fluorescens* V or VI[b]	Cucumber rhizosphere
ON5	*P. fluorescens* V or VI[b]	Lake sediment
ON30	*P. putida* A	Lake water
ON35	*P. putida* A	Lake water
DF13	*P. putida* B	Cucumber rhizosphere
DF45	*P. putida* B	Cucumber rhizosphere
ON31	*P. putida* A/B[c]	Lake sediment
DF42	*P. putida* A/B[c]	Cucumber rhizosphere
DF14	*P. putida* A/C[c]	Cucumber rhizosphere

[a] Tentative identification from biochemical characteristics.
[b] New biotype VI according to Barrett et al. (2).
[c] *P. putida* A/B and A/C were typed as *P. putida* A when classified by physiological tests. However, patterns of proteinase K-resistant whole-cell proteins from strains designated *P. putida* A/B were identical to those for *P. putida* B, and the patterns for *P. putida* A/C were intermediate between those for *P. putida* A and *P. putida* B (31).

MATERIALS AND METHODS

Bacterial strains and culture media. *P. fluorescens* DF57 (biovar III) was the main test strain used in this study. A panel of *P. fluorescens* and *P. putida* strains was used for comparison of outer membrane protein patterns (Table 1). These strains were classified by using the API 20NE system supplemented by additional tests as described by Sørensen et al. (30). Furthermore, sequence analysis of 23S ribosomal DNA has been performed for most of the strains (4).

A larger group of test strains was used to determine the specificity of the antibody. The panel of strains mentioned in Table 1 was supplemented with *P. fluorescens* DSM 50090^T, *P. putida* DSM291^T, *Pseudomonas chlororaphis* DSM 50083^T, *Pseudomonas syringae* ATCC 19310^T, *P. stutzeri* DSM 5190^T, *P. aeruginosa* DSM 50071^T, *Alcaligenes eutrophus* DSM 4058, *Escherichia coli* DSM 498, *Bacillus subtilis* DSM 402, *Shewanella putrefaciens* A27, and *Vibrio* sp. strain NCIMB 1982.

Bacteria were grown aerobically under standard conditions in 25 ml of Luria broth (1% Bacto Tryptone, 0.5% yeast extract, 1% NaCl, and 0.4% glucose). Bacteria were grown overnight at room temperature on a rotary shaker at 120 rpm.

Preparation of outer membranes and whole-cell protein. Outer membranes from the panel of *Pseudomonas* strains were isolated by the Sarkosyl method as described in reference 19. The membrane preparations were stored at −20°C after addition of 100 U of aprotinin (Sigma, St. Louis, Mo.) per ml.

In order to obtain whole-cell protein, overnight cultures were harvested and washed twice in phosphate-buffered saline (PBS) (0.025 M sodium phosphate buffer [pH 7.4], 0.125 M NaCl). The cells were resuspended in PBS, and 4 volumes of sample buffer (0.75% sodium dodecyl sulfate [SDS], 3.3% glycerol, 0.47% dithiothreitol, 0.04 μg of pyronin G, and 1 M Tris-HCl, adjusted to pH 6.8) were added. The samples were kept at −20°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Kragelund and Nybroe (19). The standard procedure was to solubilize the samples by boiling them for 5 min in sample buffer (see above). The solubilization of OprF was further tested by boiling for 1, 2, 5, and 60 min. Protein gels were either stained for 3 h with Coomassie blue R250 or silver stained by the method of Blum et al. (3). A total of 17 μg of outer membrane protein was loaded onto the gels to be stained with Coomassie blue, 10 μg of whole-cell protein was loaded onto the gels to be electroblotted (see below), and 0.5 μg of OprF was applied to each gel to be silver stained. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) as the standard.

Purification of protein from nitrocellulose paper. The outer membrane proteins of *P. fluorescens* DF57 were separated by SDS-PAGE as described above, and the protein fractions were transferred to nitrocellulose paper (BA85; Schleicher and Schuell, Dassel, Germany) by semidry electroblotting (Kem-En-Tec, Copenhagen, Denmark) at 0.8 mA/cm^2 for 2 h.

To purify OprF, its position on the electroblot was identified by staining part of the blot with 0.1% (wt/vol) naphthol blue black (Sigma) in 10% methanol–2% acetic acid and destaining with 50% methanol–7% acetic acid. An unstained area of the blot was maintained because naphthol blue black interfered with the subsequent recovery of OprF from the nitrocellulose filter. The OprF region was cut out from the unstained nitrocellulose paper, with the stained part being used as a template, and purified by elution with formic acid (17). The latter proved to be superior to elution with acetone or acetonitrile (1, 27). The recovery of protein was improved by two subsequent formic acid elution steps, whereas a third elution step did not further increase the protein recovery. The purity of the eluted protein was monitored by SDS-PAGE, and the preparations were kept at −20°C if not used immediately.

Production of antisera. Antibodies against OprF from DF57 were made by immunizing mice (strain Ssc:CF1) subcutaneously with 4 μg of purified OprF mixed (1:1) with Freund's incomplete adjuvant (State Serum Institute, Copenhagen, Denmark). Immunizations were done by the State Serum Institute according to the procedure of Harboe and Ingild (14). NaN_3 (15 mM) was added to the antisera as a preservative. Absorption of the antibody against 200 mg (wet weight) of *Xanthomonas maltophilia* per ml was performed for 20 h at 4°C.

Western blotting. After electrotransfer, the blot was blocked for 15 min in a solution containing 0.05 M Tris-HCl (pH 10.2), 0.15 M NaCl, 2% Tween 20, and 0.5% BSA. After blocking, the nitrocellulose paper was incubated overnight at room temperature with the OprF antibody diluted 1:17,500 in a solution containing 0.05 M Tris-HCl (pH 10.2), 0.15 M NaCl, 0.05% Tween 20, and 0.5% BSA. After being washed three times in washing buffer (0.05 M Tris-HCl [pH 10.2], 0.15 M NaCl, 0.05% Tween 20, 0.05% BSA), the nitrocellulose paper was reacted for 2 h with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (1:1,000 in dilution buffer) (DAKO, Glostrup, Denmark). After three washes, the color was developed with a solution containing 0.1 mg of nitroblue tetrazolium (Sigma) per ml, 0.06 mg of 5-bromo-4-chloro-3-indolylphosphate (Sigma) per ml, and 0.002 M $MgCl_2$ in 0.1 M ethanolamine (pH 9.6).

Dot immunobinding. Samples containing 10^7 cells were collected by filtration on a nitrocellulose filter. The filter was immunostained essentially as described for Western blotting. However, an incubation with PBS adjusted to pH 2.8 was included to inhibit indigenous alkaline phosphatase activity (25). In some cases, the filters were treated three times with a solution containing 0.1 M Tris-HCl (pH 9.2) and 0.01 M EDTA for 20 min at room temperature before incubation with the antibodies (33).

Immunofluorescence microscopy. Samples for immunofluorescence microscopy were filtered through 0.2-μm-pore-size black polycarbonate membranes (Nuclepore, Pleasanton, Calif.). The filters were immunostained as described above but with the following modifications: all buffers were at pH 7.6, the OprF antibody was diluted 1:1,000, and the secondary antibody was fluorescein-conjugated goat anti-mouse immunoglobulin diluted 1:20 (DAKO). Treatment with EDTA was as described above. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) as described by Hoff (15).

Plant systems. The soil used in the plant systems was a sandy loam from Højbakkegård, Tåstrup, Denmark. It was stored in plastic bags at approximately 15°C without drying. The soil was sieved (<2-mm particle size) before use. For isolation of bacteria from barley rhizosphere, plant systems were established by planting barley seeds (*Hordeum vulgare* var. Digger) in 50-ml tubes containing natural soil. The soil moisture was adjusted to 15% (wt/wt). The systems were incubated at 20°C for 10 days, with alternating 12-h periods of darkness and light.

Isolation of bacteria from barley rhizosphere. At harvest, the plants were removed from the tubes and loose soil was shaken free from the roots. After transfer to 2.5 ml of PBS per g of sample material, the rhizosphere soil was released from the roots by mild sonication in an ultrasonic bath (Metason 200; Struers) followed by vortexing for 30 s. The soil samples were spread plated in appropriate dilutions on 10% tryptic soy agar (TSA; Difco, Detroit, Mich.) (1.7% Bacto Tryptone, 0.3% Bacto Soytone, 0.25% Bacto Dextrose, 0.5% NaCl, 0.25% K_2HPO_4, 1.5% agar, and 0.005% nystatin), on King's B (KB) agar (1.0% tryptone, 1.0% Proteose Peptone no. 3, 0.15% KH_2PO_4, 0.15% $MgSO_4$, 1.0% glycerol, 1.5% agar, and 0.005% nystatin), and on freshly made Gould S1 agar (1.0% sucrose, 1.0% glycerol, 0.5% Casamino Acids, 0.1% $NaHCO_3$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.12% sodium lauryl sarcosine, 0.002% trimethoprim, 1.8% agar, and 0.005% nystatin). The plates were incubated for 48 h at 30°C. Two negative controls (*A. eutrophus* DSM 4058 and *E. coli* DSM 498) and two positive controls (*P. fluorescens* DF57 and *P. chlororaphis* ATCC 13985) were grown on each agar plate.

Colonies were transferred to nitrocellulose filters that were immunostained as described for dot immunobinding, including the EDTA treatment step, except that 1 and 0.3% casein were added to the blocking and dilution buffers, respectively. To define the cutoff level of the immunoassay, staining was stopped immediately when unspecific staining of negative controls was discernible.

A number of positive and negative colonies were identified on the blot, picked from the original plate (template), purified by two transfers to new plates, and analyzed by using the API 20NE system (Bio Mérieux SA, Marcy l'Etoile, France).

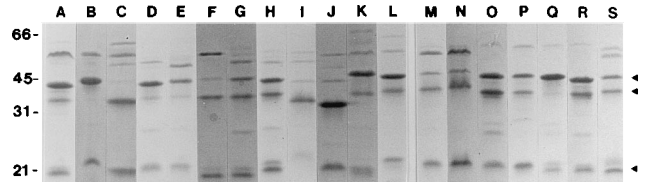

FIG. 1. SDS-PAGE profiles of *P. fluorescens* and *P. putida* outer membrane proteins. Lanes A to L, *P. fluorescens* ON24, MM5, MM6, DF8, DF57, DF61, DF23, DF18, ON13, ON25, DF17, and ON5, respectively; lanes M to S, *P. putida* ON30, ON35, DF13, DF45, ON31, DF42, and DF14, respectively. Table 1 gives further information about the strains. The lanes in the figure are from two separate gels. The gels were stained with Coomassie blue, and similar patterns were observed in three experiments. The molecular weights (in thousands) of standard proteins are shown in the margin, and the major outer membrane proteins are indicated by arrowheads.

RESULTS

Major outer membrane proteins. The heterogeneity of the major outer membrane proteins among strains representing the *P. fluorescens* and *P. putida* biovars was analyzed by SDS-PAGE. A comparison of the protein profiles revealed three conserved major outer membrane proteins. The largest of these had an M_r of 42,000 to 46,000 in the *P. fluorescens* strains (Fig. 1, lanes A to L) and an M_r of 42,000 to 44,000 in the *P. putida* strains (Fig. 1, lanes M to S), while the M_r of the second protein was 33,000 to 37,000 in the *P. fluorescens* strains and 36,000 to 38,000 in *P. putida*. The third protein, migrating at ca. 21 kDa, was often resolved into two bands at 20 and 21 kDa. These outer membrane proteins resembled the well-characterized pattern of *P. aeruginosa*: OprE (44 kDa), OprF (38 kDa), and either OprH (21 kDa) or OprL (20.5 kDa).

Besides these major proteins, three to five less predominant ones were observed (Fig. 1). In general, the protein pattern varied among the strains and the variability was greater within the *P. fluorescens* strains than within the strains representing *P. putida*. The outer membrane protein patterns were not useful for assigning the strains to a specific *P. fluorescens* or *P. putida* biovar.

Purification of the 37-kDa OprF-like protein. In *P. fluorescens* DF57 outer membranes, the OprF-like protein migrated at 37 kDa under standard conditions (see Materials and Methods) (Fig. 1, lane E). The M_r of this protein was modifiable by treatment with dithiothreitol and heat (data not shown) as reported for *P. aeruginosa* OprF (11). Electrophoretically pure OprF was obtained from outer membranes by eluting the protein from nitrocellulose membranes after electrotransfer from SDS-polyacrylamide gels (Fig. 2). The recovered protein migrated with an M_r of 39,000 when dithiothreitol was not included in the sample buffer and with an M_r of 43,000 when in the reduced form.

Specificity of the antibody directed against OprF. A polyclonal antibody against the purified OprF was raised in mice, and the specificity of this antibody was determined by Western blot analysis of whole-cell lysates. The antibody recognized a 37-kDa protein in *P. fluorescens* DF57 (Fig. 3). Additionally, the OprF antibody reacted with comparable proteins in the type strains of rRNA homology group I pseudomonads tested but not with material from a number of nonpseudomonads (Fig. 3A). It was noted that the mobility of OprF in the type strains of *P. putida*, *P. syringae*, and *P. aeruginosa* was lower than that of OprF in *P. fluorescens*, *P. chlororaphis*, and *P. stutzeri.*

When tested against the panel of *P. fluorescens* and *P. putida* strains used throughout this work, the antibody reacted with proteins with M_rs of 33,000 to 38,000 in all strains (Fig. 3B and C). The strain-to-strain variability of the apparent molecular weights of the recognized proteins corresponded to that observed for the Coomassie blue-stained proteins (Fig. 1). In some strains, additional antibody reaction to bands of higher mobility could be observed; these bands probably represented proteolytic-degradation products.

OprF localization in the outer membrane. To determine whether OprF was surface exposed in *P. fluorescens* DF57, intact cells which had not been exposed to fixatives were analyzed by immunofluorescence microscopy. In these preparations, no binding of the antibody could be detected. This negative result was assumed to be due to either a lack of antibody binding to surface-exposed epitopes or a shielding of such epitopes by LPS O side chains. To distinguish between these possibilities, cells were treated with EDTA, which has previously been found to unmask other outer membrane proteins in *P. putida* (33). This treatment dramatically improved antibody detection of OprF without affecting cell integrity, as seen in Fig. 4.

To assess whether OprF could be unmasked in all the strains included in this study, a dot immunobinding analysis of intact cells was carried out. As seen in Fig. 5A, the antibody showed only weak reactions with a few of the pseudomonads in a standard assay. However, after EDTA treatment, all pseudomonads gave a positive signal in contrast to the nonpseudomonad strains (Fig. 5B). Hence, this OprF antibody seems to be able to specifically detect rRNA homology group I pseudomonads both in Western blot analysis and in assays targeting whole cells, provided that OprF is unmasked by treatment with EDTA.

Verification of OprF-positive colonies as rRNA homology group I pseudomonads. Bacteria from rhizosphere soil were isolated on 10% TSA, Gould S1 agar, and KB agar and analyzed by colony blotting with the OprF antibody. We tested 59 OprF-positive colonies by using the API 20NE system (26 colonies were from 10% TSA, 20 were from Gould S1 agar, and 13 were from KB agar). Of the 59 colonies, 55 were identified as rRNA homology group I pseudomonads and 4 were found to be *X. maltophilia*. Also, 25 colonies which did not react with the OprF antibody were analyzed by using the API 20NE system (19 were from 10% TSA and 6 were from KB agar). None of the 25 could be identified as rRNA homology group I pseudomonads (or as *X. malthophilia*).

The reaction against *X. maltophilia* was removed by absorption with the four cross-reacting isolates without a loss of specificity to the pseudomonad type strains used throughout this study (data not shown). We therefore conclude that the colony blotting assay is specific for rRNA homology group I pseudomonads.

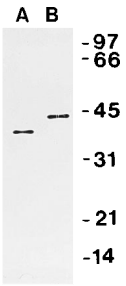

FIG. 2. SDS-PAGE of OprF protein purified from *P. fluorescens* DF57. Lane A, purified OprF, dithiothreitol not included in sample buffer; lane B, purified OprF, protein sample reduced with dithiothreitol. The gel was silver stained. The molecular weights (in thousands) of standard proteins are shown in the margin.

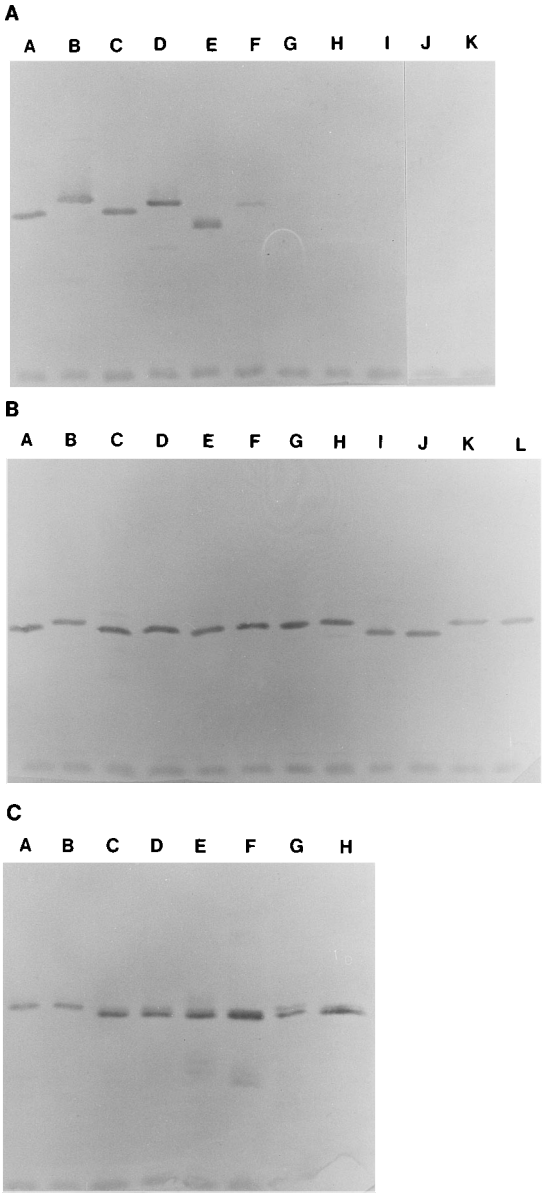

FIG. 3. Specificity of the OprF antibody tested by Western blotting. (A) Lanes A to K, *P. fluorescens* DSM 50090^T, *P. putida* DSM 291^T, *P. chlororaphis* DSM 50083^T, *P. syringae* ATCC 19310^T, *P. stutzeri* DSM 5190^T, *P. aeruginosa* DSM 50071^T, *A. eutrophus* DSM 4058, *E. coli* DSM 498, *B. subtilis* DSM 402, *S. putrefaciens* A27, and *Vibrio* sp. strain NCIMB 1982, respectively. (B) Lanes A to L, same *P. fluorescens* strains as in Fig. 1, lanes A to L. (C) Lanes A to G, same *P. putida* strains as in Fig. 1, lanes M to S. *P. fluorescens* DF57 is included for comparison in lane H. The dark band seen at the lower edge of each blot is the tracking dye, pyronin G.

Detection of rRNA homology group I pseudomonads in rhizosphere soil. Pseudomonads from rhizosphere soil were then detected by colony blotting with the absorbed OprF antibody. The percentages of OprF-positive colonies on 10% TSA, Gould S1 agar, and KB agar are shown in Table 2. All colonies on the Gould S1 agar reacted with the OprF antibody, whereas ca. 15% of the colonies on 10% TSA and ca. 9% of the colonies on KB agar were positive.

The highest estimate of OprF-containing CFU in rhizosphere soil (ca. 5×10^6 CFU/g of soil) was obtained by colony blotting from 10% TSA (Table 2). The values obtained from KB agar and Gould S1 agar were ca. 28% and ca. 93% lower, with some plant-to-plant variability.

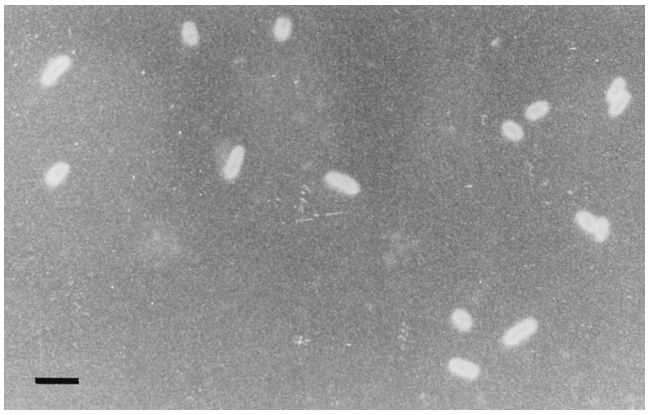

FIG. 4. *P. fluorescens* DF57 detected by immunofluorescence microscopy with OprF antibody after treatment with EDTA. Bar, 2.8 μm.

DISCUSSION

Outer membrane protein profiles. The diversity of the outer membranes of the *P. fluorescens* and *P. putida* biovars was analyzed by SDS-PAGE. Three or four major proteins with comparable mobilities were seen and could be tentatively identified as homologs to *P. aeruginosa* OprL (20.5 kDa) and/or OprH (21 kDa), OprF (33 to 38 kDa), and OprE (42 to 46 kDa) (13). In an analysis of two *P. putida* isolates and one *P. fluorescens* isolate, de Weger et al. (8) found major proteins corresponding to the ones reported here, whereas Rosello et al. (29) found major proteins of ca. 45 kDa, ca. 31 kDa, and ca. 20 kDa in *P. stutzeri.*

In spite of the conservation of some outer membrane proteins, all the *P. fluorescens* and *P. putida* isolates have unique protein profiles, and the profiles cannot be used to identify specific biovars or to distinguish between the two species. A slightly higher resolution was recently reported by Sørensen et al. (30), who used profiles of proteinase K-resistant whole-cell proteins to distinguish *P. putida* biovar A from all remaining *P putida* and *P. fluorescens* biovars. Hence, the heterogeneity of the outer membrane proteins within *P. fluorescens* and *P. putida* is larger than that described for *P. aeruginosa*, where

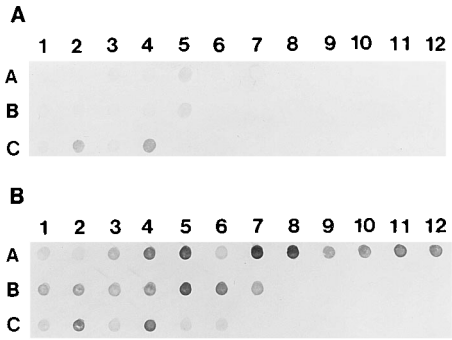

FIG. 5. Specificity of OprF antibody as tested by dot immunobinding. (A) Intact cells which had not been fixed were collected on nitrocellulose filters and incubated with OprF antibody. Dots A1 to A12, same *P. fluorescens* strains as in Fig. 3B; dots B1 to B8, same *P. putida* strains as in Fig. 3C; dots C1 to C11, same reference strains as in Fig. 3A. (B) Samples as indicated for panel A. The filter was treated with EDTA before incubation with antibodies.

TABLE 2. Detection of rRNA homology group I pseudomonads from barley rhizosphere soil by OprF-targeted colony blotting

Plant no.	Growth medium	% OprF-positive CFU	OprF-positive CFU/g of wet soil
1	10% TSA	10.2[a]	2.7×10^6
	Gould S1 agar	100[b]	2.1×10^5
	KB agar	8.7[c]	1.8×10^6
2	10% TSA	11.2[a]	3.0×10^6
	Gould S1 agar	100[b]	2.1×10^5
	KB agar	10.1[c]	2.3×10^6

[a] Total CFU included in the analysis: 401 (plant 1) and 409 (plant 2).
[b] Total CFU included in the analysis: 319 (plant 1) and 330 (plant 2).
[c] Total CFU included in the analysis: 312 (plant 1) and 358 (plant 2).

strains belonging to different serogroups have identical protein profiles (24), and for *P. stutzeri*, where outer membrane patterns of different genomovars appear to be very similar (29).

Specificity of the anti-OprF antibody. A polyclonal antibody was raised against the 37-kDa OprF from *P. fluorescens* DF57 outer membranes. In Western blot analysis, the antibody was specific to OprF from the included type strains of homology group I pseudomonads. This result is consistent with the high degree of conservation in the carboxy terminus of OprF deduced from analysis of *oprF* genes from *P. aeruginosa*, *P. syringae*, and *P. fluorescens* strains (5, 31).

Immunological evidence for conserved epitopes on OprF has been obtained through studies with monoclonal antibodies against *P. aeruginosa* OprF (20, 23). However, no individual monoclonal antibody recognizes all fluorescent pseudomonads. Finally, González et al. (9) reported only weak reactions of a polyclonal antibody produced against OprF from a psychrophilic *P. fluorescens* strain with other *P. fluorescens* strains, *P. aeruginosa*, and *Pseudomonas fragi.*

The differences in M_rs of OprF proteins from the examined type strains of homology group I pseudomonads support the notion that OprF proteins from *P. aeruginosa*, *P. putida*, and *P. syringae* have a central cysteine-rich domain which is replaced by a smaller proline-rich domain in *P. fluorescens*, *P. chlororaphis*, and *P. stutzeri* (5, 6, 20, 31). However, within the *P. fluorescens* strain panel, the M_rs of OprF proteins ranged from 33,000 (comparable to the type strain) to 37,000. The heterogeneity suggests that the proline-rich domain may not be ubiquitous within that species. This notion is supported by the purification of a dithiothreitol-modifiable OprF from *P. fluorescens* DF57, which is a typical denitrifying *P. fluorescens* strain (30).

Localization of OprF epitopes. We investigated whether the OprF antibody recognized surface-exposed epitopes, since this would be a prerequisite for detection of intact cells. Immunofluorescence microscopy demonstrated that the antibody reacted only with *P. fluorescens* DF57 cells that had been treated with EDTA. This result was extended to the entire fluorescent-pseudomonad strain panel by using a dot immunobinding screening (Fig. 5). Hence, the OprF antibody recognizes conserved surface-exposed epitopes which are, at least partly, masked by LPS. EDTA treatment was also necessary for detection of a surface-exposed PhoE-ColA marker protein in *P. putida* WCS358 by immunofluorescence microscopy, whereas the marker was readily recognized in an O-side chain-deficient mutant (33).

OprF-targeted detection of pseudomonads in rhizosphere soil. Classically, detection of fluorescent pseudomonads has been performed by enumerating either colonies on selective Gould S1 medium or fluorescent colonies on KB medium (10, 21, 32). OprF-targeted colony blotting provides a molecular specificity for rRNA group I pseudomonads, including the fluorescent species. In addition, we demonstrated that colony blotting from a general medium gives a better recovery of pseudomonads than blotting from either Gould S1 or KB medium, probably because of a higher plating efficiency (see reference 10).

We found that rRNA group I pseudomonads constitute ca. 11% of the culturable bacterial population in the barley rhizosphere. Previous reports, from studies using conventional culture techniques, have reported that fluorescent pseudomonads constitute 10 to 35% of the total bacterial population in the rhizospheres of wheat, barley, and bananas (18, 32). In contrast, Miller et al. (21) found only ca. 1% pseudomonads in the rhizospheres of maize, grass, and wheat. Hence, our results support the notion that rRNA group I pseudomonads, including the fluorescent species, are a significant component of the rhizosphere microflora.

As a large proportion of bacteria in natural environments are in a nonculturable state, it will be of interest to analyze the distribution and diversity of nonculturable rRNA group I pseudomonads. Therefore, we are currently developing methods for the detection of OprF in protein extracts from natural samples.

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