Molecular characterization of the major outer-membrane protein OprF from plant root-colonizing *Pseudomonas fluorescens*

René De Mot,¹ Geert Schoofs,¹ An Roelandt,¹ Paul Declerck,² Paul Proost,³ Jozef Van Damme³ and Jos Vanderleyden¹

Author for correspondence: René De Mot. Tel: +32 16 22 09 21. Fax: +32 16 20 07 20. e-mail: Janssens%faj%agr@CC3.KULEUVEN.AC

¹ F. A. Janssens Laboratory of Genetics, Catholic University of Leuven, Willem de Croylaan 42, B-3001 Heverlee, Belgium

² Center for Molecular and Vascular Biology, Catholic University of Leuven, Herestraat 49, B-3000 Leuven, Belgium

³ Rega Institute for Medical Research, Catholic University of Leuven, Minderbroederstraat 10, B-3000 Leuven, Belgium

N-terminal sequence analysis of peptides generated by proteolytic treatment of the *Pseudomonas fluorescens* OE 28.3 major outer-membrane protein OprF, embedded in outer membranes or present in whole cells, indicated a surface-exposed location for the proline-rich region of the protein. This region is absent from the *P. aeruginosa* and *P. syringae* OprFs. Evidence was obtained for the presence of additional exposed but less accessible regions in the carboxy half of OprF. Four OprF-specific monoclonal antibodies were all directed to the C-terminal part of the protein but did not recognize a surface-exposed epitope as shown by flow cytometry. Our data support the model previously proposed for *P. aeruginosa* OprF in which the entire protein is embedded in the outer membrane, unlike the topology proposed for the major outer-membrane protein from *Escherichia coli*, OmpA, whose carboxy half resides in the periplasmic space. For six other *P. fluorescens* strains producing OprF proteins with different isoelectric points, the primary structure was determined by sequence analysis of the PCR-amplified *oprF* genes. The proline-rich domain represented the most conserved region of the different *P. fluorescens* OprFs. Based on the sequence of its *oprF* gene, it was shown that the mushroom pathogen *P. tolaasii* is quite closely related to *P. fluorescens*. Comparative sequence analysis further showed that the carboxy half of OprF contains a sequence motif that is well conserved in the enterobacterial OmpA proteins but is also present in a number of other outer-membrane proteins, including peptidoglycan-associated lipoproteins.

Keywords: *Pseudomonas fluorescens*, major outer-membrane protein, OprF

INTRODUCTION

Plant root colonization by bacteria is a complex process in which constituents from roots, bacteria and soil interact. This phenomenon is considered crucial for enabling so-called plant growth-promoting rhizobacteria, of which certain fluorescent *Pseudomonas* species are the most prominent representatives, to exert their beneficial effect on the host plant (Lugtenberg *et al.*, 1991; O'Sullivan & O'Gara, 1992).

From the cell envelope of the wheat-rhizosphere-

colonizing strain *P. fluorescens* OE 28.3, we purified a protein displaying *in vitro* adhesion to plant roots (De Mot & Vanderleyden, 1991). Subsequent cloning of the structural gene revealed a strong homology with the constitutive major outer-membrane protein (OprF) from the opportunistic human pathogen *P. aeruginosa* (Duchêne *et al.*, 1988) and the plant pathogen *P. syringae* (Ullstrom *et al.*, 1991). However, the *P. fluorescens* OprF differed substantially in the central domain where a conspicuous proline-rich repeat replaced the cysteine-rich part that is highly conserved in *P. aeruginosa* and *P. syringae* (De Mot *et al.*, 1992). A structural role has been assigned to the *P. aeruginosa* OprF, similar to the enterobacterial OmpA protein (Gotoh *et al.*, 1989a, b; Woodruff & Hancock, 1989). In addition, pore-forming activity has been reported for both proteins (Nikaido *et al.*, 1991; Bellido *et al.*, 1992; Sugawara & Nikaido, 1992), though lower than

The GenBank accession numbers for the sequences of the *oprF* genes from *P. fluorescens* strains NRRL B-15132, PGSB 7941, PGSB 8059, PGSB 8472, OE 46.1, and from *P. tolaasii* CH 36 reported in this paper are L21197, L21198, L21199, L21200, L21202 and L21201, respectively.

Table 1. *Pseudomonas* strains used in this study

Strain*	Relevant characteristic	Source or reference†
<i>P. aeruginosa</i> PAO1		D. Haas, ETH
<i>P. aureofaciens</i> LMG 1245 ^T	Isolate from river clay (The Netherlands)	LMG
<i>P. cepacia</i> [II] RW5P1	Isolate from rice rhizosphere (Sri Lanka)	Vlassak <i>et al.</i> (1992)
<i>P. cichorii</i> LMG 2162 ^T	Pathogen of chicory (Germany)	LMG
<i>P. chlororaphis</i> LMG 5004 ^T	Plate contaminant	
<i>P. diminuta</i> [IV] LMG 2089	Fresh water stream (USA)	
<i>P. facilis</i> [III] LMG 2193 ^T	Soil isolate (USA)	
<i>P. fluorescens</i>		
OE 28.3	Isolate from wheat rhizosphere (Belgium)	De Mot & Vanderleyden (1991)
OE 46.1	Isolate from maize rhizosphere (Belgium)	H. Joos, PGS
PGSB 7941	Isolate from maize rhizosphere (France)	
PGSB 8059	Isolate from maize rhizosphere (France)	
PGSB 8472	Isolate from maize rhizosphere (France)	
NRRL B-15132	Isolate from wheat rhizosphere (USA)	Weller & Cook (1983)
NRRL B-15134	Isolate from wheat rhizosphere (USA)	Weller & Cook (1983)
VA 2.1	Isolate from wheat rhizosphere (Belgium)	H. Joos, PGS
ZA 4.1	Isolate from wheat rhizosphere (Belgium)	
PGSB 7828	Isolate from wheat rhizosphere (Belgium)	
PGSB 8059	Isolate from maize rhizosphere (Belgium)	
PGSB 8273	Isolate from maize rhizosphere (Belgium)	
PGSB 7861	Isolate from maize rhizosphere (Belgium)	
<i>P. mendocina</i> LMG 1223 ^T	Soil isolate	LMG
<i>P. putida</i> RW1P1	Isolate from rice rhizosphere	Vlassak <i>et al.</i> (1992)
<i>P. stutzeri</i> LMG 1228		LMG
<i>P. syringae</i> pv. <i>syringae</i> LMG 1247 ^R	Pathogen of <i>Syringa vulgaris</i> (UK)	
<i>P. tolaasii</i> CH 36	Pathogen of <i>Agaricus bisporus</i> (Belgium)	F. Leyns, PGS
<i>P. syringae</i> pv. <i>glycinea</i> LMG 5066 ^R	Pathogen of <i>Glycine max</i> (New Zealand)	LMG
<i>P. viridiflava</i> LMG 2352 ^T	Isolate from <i>Phaseolus</i> sp. (Switzerland)	
<i>X. maltophilia</i> [V] PGSB 1894		H. Joos, PGS

* The strains all belong to the *Pseudomonas* rRNA group I, unless indicated otherwise (roman numerals in square brackets after species name). Type strains or pathovar reference strains are labelled ^T or ^R, respectively.

† Abbreviations used: ETH, Eidgenössische Technische Hochschule, Zürich, Switzerland; LMG, Culture Collection of Laboratory of Microbiology, University of Gent, Gent, Belgium; PGS, Plant Genetic Systems, Gent, Belgium.

with most porins. Although OmpA and OprF proteins are homologous in their C-terminal regions, different membrane topologies have been proposed (Vogel & Jähnig, 1986; Finnen *et al.*, 1992; Wong *et al.*, 1993).

In this paper, the topology of the OprF from *P. fluorescens* OE 28.3 was investigated using protease treatment of membrane-embedded OprF. In addition, the extent of inter-strain variation in the primary sequence of OprF from several rhizosphere isolates of *P. fluorescens* was analysed.

METHODS

Bacterial strains and culture conditions. The bacterial strains used in this work are listed in Table 1. The *Pseudomonas* strains were grown in 3% trypticase soy broth (TSB) at 30 °C.

Immunological techniques. Purified OprF protein (10 µg) mixed with Freund's complete adjuvant was used to immunize BALB/c mice subcutaneously. After 2 and 6 weeks, another 10 µg of antigen in Freund's incomplete adjuvant and in physiological salt solution, respectively, were injected intra-

peritoneally. Two days after the last injection, an intraperitoneal booster dose (10 µg) was administered. Two days later, spleen lymphocytes were isolated and fused with mouse myeloma cells P3X63-Ag8-6.5.3. Hybridomas were selected in hypoxanthine-aminopterin-thymidine-containing medium and the supernatants were screened for specific antibody production by ELISA in microtitre plates coated with purified OprF protein, using rabbit anti-mouse IgG-peroxidase (Bio-Rad) for detection. Positive hybridomas were cloned by limiting dilution. Ascitic fluid from pristane-primed mice injected with the respective hybridomas was used for purification of IgG by affinity chromatography on protein A-Sepharose (Pharmacia Biotech). Four monoclonal antibodies thus obtained (MA-28B9, MA-29A6, MA-40D1 and MA-8F5) were used in this study. Immunodetection after Western blotting was carried out with goat anti-mouse IgG-conjugated alkaline phosphatase (Harlowe & Lane, 1988). For whole-cell ELISA, the method of Charbit *et al.* (1991) was adopted. Microtitre plate wells were coated with a suspension of about 5×10^7 cells and incubated with primary antibody at 10 µg ml⁻¹. FITC-labelled rabbit anti-mouse IgG (SeroTec) was used to monitor whole cell binding of antibodies by flow cytometry as described by Srikumar *et al.* (1992). In these experiments, a mutant lacking OprF constructed

by reverse genetics (R. De Mot, unpublished data) was used as a negative control.

Sequencing of PCR-amplified *oprF* genes. Two sets of PCR primers, based on the sequence of *P. fluorescens* OE 28.3 *oprF*, were designed to amplify two overlapping fragments of the *oprF* genes from total DNA of selected fluorescent *Pseudomonas* strains. For the N-terminal regions, OLIPCR1: 5' *AAGAA-TTCAGATGGGGATTAAACGGATGAAACTG* 3'; and OLIPCR3: 5' *AAGGATCCTTRTCGAARTCGAAYTTNACRTC* 3' (where R = A, G; Y = C, T; and N = A, G, C, T) were used. The forward primer (OLIPCR1) covers a perfectly conserved sequence around the ATG start (in bold) of the *oprF* genes from *P. aeruginosa*, *P. fluorescens* and *P. syringae*. The reverse primer (OLIPCR3) reflects the OprF consensus sequence DVKFDGDK, taking the codon usage in the *P. fluorescens* *oprF* (De Mot *et al.*, 1992) and *recA* (De Mot *et al.*, 1993) genes into consideration to reduce the extent of degeneration. The C-terminal parts were amplified with OLIPCR5: 5' *ATTAGG-ATCCGGYYTGGGYGTNAACTTCGGYGG* 3'; and OLIPCR6: 5' *ATTAGAAATTCYAYTKNGCYTSNGCYTCNAC* 3' (where K = G, T; and S = G, C). The consensus sequence GLGVNFGG for the six strains deduced from the respective N-terminal PCR products was used to design the forward primer OLIPCR5, whereas the reverse primer OLIPCR6 corresponds to the C-terminal consensus sequence VEA[Q,E]A[Q,K] plus stop codons. After a hot start at 94 °C, 25–30 cycles of amplification were performed with template denaturation at 94 °C for 1 min, primer annealing at 50 °C for 2 min, and elongation at 72 °C for 3 min, concluded with a final 10 min elongation step at 72 °C. *Pfu* polymerase (Stratagene) was preferred over the *Taq* enzyme for its higher fidelity in DNA synthesis (Arnheim & Erlich, 1992). Appropriate PCR fragments were purified from agarose gels by the double GeneClean protocol (BIO101), digested with *EcoRI* and *BamHI* to generate cohesive ends within the incorporated primer tags (shown in *italic*), and cloned in plasmid pUC19. Double-stranded DNA sequencing was carried out using the AutoRead Sequencing Kit (Pharmacia Biotech) on an automated ALF sequencer (Pharmacia Biotech). For each strain, at least three independent clones were analysed. Sequence data were processed using the ASSEMBLER program (PC Gene: IntelliGenetics). The PC Gene software was also used for multiple alignments of DNA and protein sequences (CLUSTAL; Higgins & Sharp, 1988).

Electrophoretic techniques. Protein extracts for one- or two-dimensional electrophoresis were prepared by phenol extraction of whole cells or outer-membrane preparations (De Mot & Vanderleyden, 1989). SDS-PAGE was carried out in a vertical Laemmli system with a 15% or 18% polyacrylamide separating gel. Two-dimensional PAGE was carried out as described previously (De Mot & Vanderleyden, 1989). For semi-dry blotting at alkaline pH onto polyvinylidene difluoride (PVDF) membranes (Millipore) the procedure of Matsudaira (1989) was used.

Proteolysis of membrane-associated OprF. OprF-enriched outer-membrane fraction (30 µl) from strain OE 28.3, prepared as described previously (De Mot & Vanderleyden, 1991), was suspended in 1 ml 30 mM Tris/HCl, pH 8.1, or 30 mM Na₂HPO₄/NaH₂PO₄ buffer, pH 6.2. Digestion with pronase E (100 µg ml⁻¹; Sigma), proteinase K (100 µg ml⁻¹; Sigma), both at pH 8.1, and papain (50 µg ml⁻¹; Sigma) at pH 6.2 was carried out at 37 °C for 2 h. PMSF was then added to 2.5 mM. The outer membranes were reisolated and washed twice in 30 mM Tris/HCl buffer, pH 8.1, by ultracentrifugation (50 000 g) for 30 min at 4 °C. The remaining membrane-associated peptides were then electrophoretically separated and blotted onto PVDF membranes for N-terminal micro-

sequencing by automated Edman degradation using a 477A Protein Sequencer (Applied Biosystems). In a similar experiment, washed *P. fluorescens* cells were resuspended in 0.1 M sodium phosphate buffer, pH 6.2, at about 10¹⁰ cells ml⁻¹ and incubated with papain (500 µg ml⁻¹) for up to 1 h at 25 °C. Subsequently, outer membranes were isolated from the protease-treated cells in the presence of PMSF and analysed as described above. Identification of OprF-derived peptides was facilitated by the protein's relative abundance in the outer-membrane preparations. Low molecular mass calibration proteins (Fluka) were used to estimate the size of the different peptides.

RESULTS

Isolation of OprF-specific monoclonal antibodies for *P. fluorescens* OE 28.3

The specificity of the four monoclonal antibodies isolated (MA-28B9, MA-29A6, MA-40D1 and MA-8F5) was investigated by Western blotting of total cell proteins from several *P. fluorescens* strains and a number of related or more distant species (data not shown). These antibodies displayed a highly similar pattern of reactivity for the strains tested (listed in Table 1). Since no qualitative differences in reactivity were observed with peptides generated by different proteases (see below), it appeared that the respective epitopes, if not identical, are all confined to a limited area of the protein.

The banding pattern of *P. fluorescens* OE 28.3 was detected in all the *P. fluorescens* strains tested, except for the non-reacting strain PGSB 7941. Among the other *Pseudomonas* species representing the five rRNA homology groups, a weak cross-reactivity was only observed with *P. aeruginosa* and *P. syringae* pv. *syringae*, both producing a band of slightly higher apparent molecular mass, as expected for their OprFs (Duchêne *et al.*, 1988; Ullstrom *et al.*, 1991). No epitopes were recognized in more distant rhizosphere bacteria from the genera *Agrobacterium*, *Azospirillum*, *Erwinia*, *Phyllobacterium* and *Rhizobium*.

Whole-cell ELISA for the strains listed in Table 1 indicated a weak reactivity of the above positive strains with the monoclonal antibodies. However, these results could not be confirmed by flow cytometry, indicating that the epitopes are weakly surface-exposed or inaccessible in intact cells. The presence of lipo- or exopolysaccharides may interfere with antibody binding to the outer-membrane protein in the intact cells.

Protease treatment of membrane-embedded OprF

Digestion of membrane-associated OprF from *P. fluorescens* OE 28.3 by papain, proteinase K and pronase E, produced only a limited number of peptides (Fig. 1a). The differences in band intensities probably reflected differences in relative rates of hydrolysis and/or accessibility of the respective sites. N-terminal sequence analysis of these peptides enabled unambiguous identification of several cleavage sites, since the amino acid sequence of OprF from strain OE 28.3 is known (see Fig. 3 for the sequence). The data compiled in Table 2 and Fig. 1(e) show that primary cleavage sites for the three proteases were located between amino acids 187 and 191 of the mature protein,

Fig. 1. Peptide profiles generated by treatment of OprF-enriched outer membranes (a and b) and of whole *P. fluorescens* cells (c and d) with pronase E (E), proteinase K (K) and papain (P), analysed by SDS-PAGE (15% gel for a and b; 18% gel for c and d). Control samples (untreated) are labelled U. The gels or blots were stained with Coomassie blue (a and c) or immunostained with MA-28B9 (b and d). OprF (33 kDa) and its heat-modified form (39 kDa) are marked with an arrow and an asterisk, respectively. Numbering of the peptide bands is the same as used in Table 2. The time-course of OprF hydrolysis in whole cells by papain is shown in (d) with sampling after 5, 15, 30 and 45 min as indicated. A summary of the identified peptides (bands 1–10) is presented in (e). The estimated molecular mass of these peptides is indicated on the right. The scale bar represents the numbering of amino acids in mature OprF of strain OE 28.3. The shaded region of this protein represents the proline-rich sequence.

right behind the peculiar proline-rich repeat of OprF. After cleavage, both the N- and C-terminal parts remained associated with the membrane. The larger N- and C-terminal peptides of 20 kDa (band 1) and 14 kDa (band 7), respectively, accounted for the full-size OprF protein (32104 Da). The smaller (18 kDa) N-terminal peptide (bands 2, 4 and 6) was probably generated by a second cleavage of the 20 kDa peptide (band 1) a few amino acids ahead of the first site, presumably located just in front of the proline-rich stretch (calculated size: 17.5 kDa). The presence of additional cleavage sites closer to the C-terminus of the 14 kDa peptide (band 7) can explain the appearance of the smaller minor bands of about 13 kDa (bands 3 and 5), 12 kDa (band 8) and 9 kDa (band 9), having the same N-terminal end. The latter peptide was only poorly visible upon Coomassie blue staining but was clearly demonstrated by Western blotting (Fig. 1b). Sequence analysis of band 3 (pronase E) and band 5 (proteinase K) revealed some microheterogeneity due to cleavage at adjacent positions within the QVVRVE sequence (Table 2).

From this it can be concluded that the regions around residues 165 and 191 constituted exposed domains, whilst the rest of the molecule, especially the N-terminal region, was well protected by the outer-membrane environment. It appeared that this accessible region did not face the periplasmic space but was located at the cell surface, since a similar peptide pattern was obtained with papain-treated whole cells (Fig. 1c). Under these conditions, however, an additional cleavage product was detected containing the proline repeat at its N terminus (band 10). The apparent molecular mass of the latter peptide (about 23 kDa) was unexpectedly high. Assuming that no additional cleavage had occurred in the C-terminal part, the molecular mass calculated from the amino acid sequence is much lower (14.6 kDa). This discrepancy may be due to the presence of the unusual proline-rich stretch at its N-terminal end which imposes an extended coil structure (De Mot *et al.*, 1992) and may have reduced the electrophoretic mobility of the peptide. Since this fragment was not generated from protease-treated outer membranes, the accessibility of this region may differ in the native configuration

Table 2. Survey of outer-membrane-associated peptides generated by protease treatment of OprF from *P. fluorescens* OE 28.3 present in outer-membrane preparations or in whole cells (the latter with papain only)

Enzyme	Peptide			
	Band*	N-terminal sequence†	Molecular mass (kDa)	Immuno-reactivity‡
Pronase E	1	¹ QGQG	20	—
	2	¹ QGQ	18	—
	3	¹⁹⁰ RVEL§	13	+
		¹⁹¹ VELD		
Proteinase K		¹⁸⁹ VRVE		
	4	¹ QGQGAVEGELF	18	—
	5	¹⁸⁸ VVRVELDVKFD	13	+
Papain		¹⁸⁹ VVRVELDVKFD		
	6	¹ QGQG	18	—
	7	¹⁸⁸ VVRVEL	14	+
	8	¹⁸⁸ VVRVELDVKF	12	+
	9	¹⁸⁸ VVRVEL	9	+
	10¶	¹⁶⁶ APATP	23	+

* Same numbering as in Fig. 1.

† The superscript at the N-terminal amino acid refers to its position in mature OprF.

‡ A similar immunoreactivity pattern was obtained with MA-28B9, MA-29A6, MA-40D1 and MA-8F5.

§ Predominant peptide in band 3.

|| Equal amounts present in band 5.

¶ Only detectable with whole cells.

(whole cells) as compared to its configuration in isolated membrane fragments.

All immunoreactive bands contained C-terminal peptides, the smallest being the minor 9 kDa fragment (Fig. 1b and Table 2). Given this size, the epitopes should be located somewhere between position 188 and 270. To gain insight into the relative susceptibility of the respective target sites, the time-course of OprF hydrolysis by papain in whole cells was analysed by Western blotting (Fig. 1d). After 5–15 min, the main immunodetectable product was band 7, whereas only a low amount of band 10 was visible. At this early stage, two intermediate degradation products were generated (located between OprF and band 10), most likely by C-terminal cleavage. Their further degradation coincided with the appearance of the 9 kDa peptide (band 9). The minor 12 kDa peptide appeared only upon prolonged incubation.

Strain-dependent differences in isoelectric point of OprFs

Two-dimensional electrophoresis of cellular proteins from the immunoreactive strains showed that they contained a major protein with a similar relative molecular mass to OprF from strain OE 28.3. However, Fig. 2 clearly shows a difference in isoelectric point (pI) between the OprF from strain OE 28.3 (type A; Fig. 2a) and the more acidic OprF from strain NRRL B-15132 (type C;

Fig. 2c). The type A OprF was also present in strains OE 46.1 and PGSB 8472, whereas type C also occurred in strains VA 2.1, ZA 4.1, PGSB 7828, PGSB 8059, PGSB 8273, NRRL B-15134 and PGSB 7861 (data not shown). Strain PGSB 7941 contained a less acidic OprF-like protein (type B; Fig. 2b) which did not react with our monoclonal antibodies. Surprisingly, this was also the case for the mushroom pathogen *P. tolaasii* CH 36. Confirmation that the major spot obtained from *P. tolaasii* cells represented an authentic OprF protein was obtained by N-terminal sequence analysis: the first 38 amino acids were identical to the N-terminus of strain OE 28.3 OprF.

PCR amplification and sequencing of *oprF* genes

To compare the differences in primary structure between the three types of OprF identified above, the *oprF* genes of six representative strains (two of each pI type) were cloned after PCR (see Methods) and sequenced. PCR primers were based on conserved regions present in the OprF proteins from *P. aeruginosa*, *P. fluorescens* and *P. syringae*. This enabled cloning of almost the entire *oprF* coding regions from *P. fluorescens* strains OE 46.1 and PGSB 8472 (type A), NRRL B-15132 and PGSB 8059 (type C), PGSB 7941 (type B), and *P. tolaasii* CH 36 (type B). The *in vitro* amplified DNA fragments included the signal peptide region (except for the first two amino acids), but not the six C-terminal amino acids (Fig. 3). For

Fig. 2. Two-dimensional electrophoresis of total cell proteins from *Pseudomonas* strains containing an OprF protein (marked by arrow) with a pI higher (b; CH 36) or lower (c; NRRL B-15132) than *P. fluorescens* OE 28.3 OprF (a). The pH gradient extends from left (basic) to right (acidic). The migration distances of the molecular mass calibration proteins (kDa) are indicated on the right.

the PGSB 8059 OprF, 21 additional residues at the C-end were lacking because of the presence of a *Bam*HI site in the *oprF* gene.

Fig. 3 shows that the signal peptides of the different OprF proteins were highly similar and strongly resembled the

corresponding part of the unprocessed OprF from *P. syringae* (Ullstrom *et al.*, 1991). For the mature OprFs, the seven sequences showed 83.3% identity over 275 residues, and 27 of the 46 non-conserved residues contained at least one substitution with a dissimilar residue. From Fig. 3 it is also apparent that the extent of primary sequence variation differed along the length of the OprFs. The overall inter-strain variability appeared to be lower in the C-terminal half. The best conserved region was found between residues 142 and 219 and contains the proline-rich sequence (residues 167–184). Substitutions of charged residues by non-charged ones and vice versa were spread throughout the sequences and appeared not to be confined to well delineated regions of the three OprF types.

In the C-terminal region spanning residues 221–230, the OprFs from strain CH 36 and PGSB 7941 (KQYPQT-TTVVE) differed substantially from the other five proteins with their consensus sequence AQYP[A,D]TNV-EVA (Fig. 3). Actually, in this part these two type B OprFs were more similar to the corresponding OprF sequence from *P. aeruginosa* (KQYPSTSTTVE) and of *P. syringae* (QQYPQTTTVE). This region, located within the boundaries of the 9 kDa immunoreactive peptide, is a likely candidate for the epitope of the monoclonal antibodies, since the OprFs from strains CH 36 and PGSB 7941 were non-reactive, whereas the OprFs from *P. aeruginosa* and *P. syringae* showed a weak cross-reactivity (see above). In the model proposed for the membrane topology of *P. aeruginosa* OprF, the equivalent region of this putative epitope is located on the periplasmic side of the outer membrane (Wong *et al.*, 1993). Assuming a similar topology for this region of *P. fluorescens* OprF, this may explain the lack of reactivity of the monoclonal antibodies with whole cells (see above).

In the OprF-based dendrogram, strains CH 36 and PGSB 7941 clearly constitute a separate cluster (Fig. 4). Since strain CH 36 is a virulent strain of *P. tolaasii* causing toxin-mediated disease symptoms (brown blotch) on mushroom caps and producing the highly specific white-line reaction with '*Pseudomonas reactans*' (Wong & Preece, 1979; Goor *et al.*, 1986), *P. fluorescens* PGSB 7941 was also tested for, but was found not to express these phenotypes (data not shown). The clustering of the five other strains of *P. fluorescens* did not completely reflect the differences observed in pI of their respective OprFs: strain OE 46.1 (type A) clustered with PGSB 8059 (type C) but was less closely related to strains OE 28.3 and PGSB 8472 (both type A). Fig. 4 also clearly illustrated the relative distance between the *P. fluorescens* strains and *P. aeruginosa*/*P. syringae*.

The primer combination OLIPCR1–OLIPCR3 was used to amplify *oprF*-like sequences from other members of the *Pseudomonas* rRNA group I, including *P. cichorii*, *P. chlororaphis* and *P. viridiflava* (data not shown). This is not unexpected since earlier reports based on cross-reactivity of antibodies (Mutharia & Hancock, 1985) and on DNA hybridization (Ullstrom *et al.*, 1991) indicated the occurrence of *oprF*-like genes and their products in several species of this group.

OE 28.3	MKLKNTLGFAIGSIIAATSPGALA/QGGGAVEGELFYKKQYNDSVKHIEDGFNPG										30
PGSB 8059	---	L	L	V							
OE 46.1	---	L	L	V							
NRRL B-15132	---	L	L	V							
PGSB 8472	---	L	TL			Q		F		Y	
CH 36	---	L	TL								
PGSB 7941	---		L			Q					
		*		*		*					
						-					
OE 28.3	ARIGYFLTDDLSLNLSDYDKTNHTRSDGTGSSQKIGGDTSSSLTAQYHFGQAGVDSLRPYVE										90
PGSB 8059						N	K	G	V		
OE 46.1							K	G	V		
NRRL B-15132						N	K	G	V		
PGSB 8472	S	I	V	V	N	T	N	K	G	V	
CH 36							N	G			
PGSB 7941	S		V	I	N	T		K	G	V	
	*				*		*	*	*	*	
	-				-		-				
OE 28.3	GGFGHQSRGNVVKADGHSGRQDQSTLAIAGAGVKYYFTNNVYARAGVEADYALDNGKWDYSA										150
PGSB 8059		T	Q			T	F	T	V	T	
OE 46.1			Q				T	F	T	V	
NRRL B-15132		T	V			T	F	I	T		
PGSB 8472		T	Q						LF		
CH 36		S	E		K				L		
PGSB 7941		T	EV		K				LF		
		*	**		*		*	*	*	*	
		-			-						
OE 28.3	LVGLGVNFGGNAGAAAPAPTPAPAPEPTPEPEAPVAGVVRVVELDVKFDKSVVKPNSYG										210
PGSB 8059										A	
OE 46.1											
NRRL B-15132						V					
PGSB 8472											
CH 36											
PGSB 7941						V					
						*					
OE 28.3	DVKNLADFMAQYPATNVEVAGHTDSIGPDAYNQKLSQRRADRVKQVLVKDGVAPSRTITAV										270
PGSB 8059						V		A		V	
OE 46.1						V		A		V	
NRRL B-15132			D			V		A		S	
PGSB 8472								A			
CH 36		K	Q	TTV	E	V		A		I N VSS	
PGSB 7941		K	Q	TTV	E	V		A		I N VSS	
		*		*	*	*		*		*	
		-		-	-	-		-			
OE 28.3	GYGESRPVADNATEAGRAVNRNRVEASVEAQAQ										302
PGSB 8059	G	-----	-----	-----	-----	-----	-----	-----	-----	-----	
OE 46.1											
NRRL B-15132						A	-----	-----	-----	-----	
PGSB 8472							-----	-----	-----	-----	
CH 36	K				I		-----	-----	-----	-----	
PGSB 7941	K				I		-----	-----	-----	-----	
	*										

Fig. 3. Alignment of the deduced OprF sequences for six *P. fluorescens* strains and *P. tolaasii* CH 36. Using strain OE 28.3 OprF as a reference, only residues differing in the other proteins are shown. The numbering refers to the position in the mature OprF (with every tenth residue marked by a vertical line). Variable positions with substitutions involving non-similar residues are marked by asterisks. Among these, the ones involving a change in charge are underlined. The signal peptides are shown in italics (slash indicates processing site). Experimentally determined amino acid sequences for strain OE 28.3 OprF from strains CH 36 (1–38) and PGSB 8472 (1–43). Dashes indicate a lack of sequence data. Identified cleavage sites in membrane-protected OprF from strain OE 28.3 for papain (a), papain and proteinase K (b), proteinase K and pronase E (c), and pronase E (d) are marked by labelled open arrowheads. Residues corresponding to perfectly conserved positions within the proposed OmpA signature (see text) are underlined twice.

Homology of OprF with OmpA and OmpA-related proteins

Previously, we and others pointed out that several outer-membrane proteins bear considerable sequence homology in their respective C-terminal parts to the corresponding domains of OmpA proteins from Enterobacteriaceae: the

Neisserial protein III and class-4 proteins (Gotschlich *et al.*, 1987; Klugman *et al.*, 1989), the OprFs from *Pseudomonas* species (Duchêne *et al.*, 1988; Ullstrom *et al.*, 1991; De Mot *et al.*, 1992) and a 21 kDa protein from *Bordetella avium* (Gentry-Weeks *et al.*, 1992). A further database search for additional related sequences revealed that the peptidoglycan-associated lipoproteins (PALs)

Fig. 4. OprF-based dendrogram for six *P. fluorescens* strains and *P. tolaasii* CH 36 constructed by multiple alignment of 296 amino acid residues (CLUSTAL program). To enable inter-species comparison, the equivalent portion of the OprFs from *P. aeruginosa* ATCC 33354 and *P. syringae* ATCC 19310 were included.

from *Escherichia coli* (Chen & Henning, 1987), *Haemophilus influenzae* (Deich *et al.*, 1988; Nelson *et al.*, 1988) and *Legionella pneumophila* (Engleberg *et al.*, 1991) should be added to this list, as well as a putative lipoprotein from *E. coli* (Byström *et al.*, 1983). Recently, three additional *Haemophilus* outer-membrane proteins with C-terminal sequence homology to OmpA were described: a haemolytic 31 kDa protein from '*Haemophilus somnus*' (Won & Griffith, 1993) and two *H. influenzae* proteins sharing 92% identity, namely protein P5 (Munson *et al.*, 1993) and a non-typeable fimbrial protein (GenBank L08448). Strong sequence homology in the C-terminal part of the major outer-membrane protein CD of *Branhamella catarrhalis* with the OprF proteins from *Pseudomonas* species was reported by Murphy *et al.* (1993).

A multiple alignment was performed for these sequences, including the data from Lawrence *et al.* (1991) deduced from PCR-amplified and sequenced *ompA* genes from several additional enterobacterial species, and the data presented in this paper for OprF proteins from several *Pseudomonas* strains. A striking local sequence homology was apparent in the C-terminal part of the aligned sequences and the following consensus sequence was deduced: GX[T,A][D,N,S]X₂[G,S]X_{3,6}[Y,F,A]NX₂L-[S,G][E,Q,L]XRAX₂V (where X denotes more variable positions) (see positions 231–253 in Fig. 3). In the SwissProt 26 database, no additional proteins were found which contained the sub-motif [Y,F,A]NX₂L[S,G]-[E,Q,L]XRAX₂V. Therefore, this region may be useful as a fingerprint for identification of OmpA-related proteins. In *P. fluorescens* OE 28.3, this region is contained within the 9 kDa peptide (band 9) and so appears inaccessible to proteases (Table 2 and Fig. 3).

DISCUSSION

Proteolysis of membrane-bound OprF from *P. fluorescens* OE 28.3 revealed that most of the protein's domains were not accessible to pronase, proteinase K and papain, and that the N-terminal half of the protein had no cleavage site. However, these enzymes all cleaved OprF at one or

more sites adjacent to the proline-rich repeat in both isolated membranes and whole cells. Therefore, this region appears to be freely exposed to the cell surface. Based on its particular amino acid composition, we previously proposed that this region may reside outside the outer membrane (De Mot *et al.*, 1992). The observation that the exposed proline-rich sequence itself is not proteolytically cleaved, is not surprising since such activity is limited to specific proteases acting on short peptides only (Szwajcer-Dey *et al.*, 1992). Three additional protease sites were demonstrated in the C-terminal half but apparently these were less frequently cleaved, probably because of steric hindrance. Also for the OprF from *P. aeruginosa*, evidence suggested that surface-exposed regions were present in the C-terminal part (Finnen *et al.*, 1992; Hughes *et al.*, 1992). The role of the conspicuous proline repeat in the *P. fluorescens* OprF remains to be elucidated and some functional importance may be anticipated from its high degree of inter-strain conservation. In general, exposed loops of outer-membrane proteins are the more variable regions. Remarkably, such an extended proline stretch is absent from the otherwise closely related OprFs from *P. aeruginosa* and *P. syringae*, and is replaced with a larger disulphide-stabilized domain (Finnen *et al.*, 1992). This cysteine domain is thought to be exposed on the bacterial surface (Wong *et al.*, 1993). In the OmpA proteins of at least ten enterobacterial species a similar though shorter proline stretch is very well conserved, albeit the topology of the adjacent C-terminal region appears to be quite different from the one assigned to OprF (see below). Furthermore, such a proline-rich sequence is also present in the OmpA-related proteins from *Neisseria* species (Gotschlich *et al.*, 1987; Klugman *et al.*, 1989). Attempts to elucidate the role of the four-fold alanine-proline repeat in *E. coli* OmpA by mutagenesis, have shown that its elimination does not impair incorporation into the outer membrane (Klose *et al.*, 1988a), but no function was assigned to it.

Our data indicate that the OprF protein from *P. fluorescens* OE 28.3, including most of the C-terminal part, is embedded in the outer membrane. This is in agreement with the model proposed for *P. aeruginosa* OprF (Finnen *et al.*, 1992; Wong *et al.*, 1993). Considering the extent of homology between OmpA and OprF in the carboxy halves of these proteins, this membrane topology for the *Pseudomonas* proteins is quite intriguing. Indeed, there is ample evidence that the OmpA carboxy half rests in the periplasm and is not essential for membrane insertion (Morona *et al.*, 1984; Vogel & Jähnig, 1986; Klose *et al.*, 1988a, b; Surrey & Jähnig, 1992). Both models could be reconciled assuming that the highly conserved C-terminal region plays a role not in membrane incorporation but in the interaction with the peptidoglycan layer. This would then account for the high level of localized sequence conservation between widely different species of a set of proteins, apparently quite diverse in size, function and topology.

The identification of surface-exposed loops in outer-membrane proteins not only contributes to a better understanding of their topology and function, but also

offers new perspectives for the engineering of Gram-negative bacteria. Indeed, such surface domains can be used to target heterologous proteins or parts thereof to the outer membrane, thereby anchoring novel polypeptides onto the bacterial surface. Potential applications of such surface-targeted expression have been highlighted in recent reviews (Georgiou *et al.*, 1993; Little *et al.*, 1993).

Sequence analysis of *in vitro* amplified genes encoding outer-membrane proteins has already proven its value to pinpoint inter-species and inter-serovar differences at the amino acid level among strains of human pathogens, such as *Chlamydia* and *Neisseria* species (Butt *et al.*, 1990; Hayes *et al.*, 1990; Maiden *et al.*, 1991; Dean *et al.*, 1992; Kaltenboeck *et al.*, 1993; Lampe *et al.*, 1993). Lawrence *et al.* (1991) showed that such sequence data are quite useful for phylogenetic analysis of closely related bacteria such as enterics. Application of this approach to the *oprF* genes from several fluorescent *Pseudomonas* strains, revealed a close relationship of the mushroom pathogen *P. tolaasii* with typical rhizosphere isolates of *P. fluorescens*, including the well known biocontrol strain NRRL B-15132 (= 2-79) (Weller & Cook, 1983; Mazzola *et al.*, 1992). These molecular data strongly support the view that *P. tolaasii* is a typical member of the *Pseudomonas* RNA homology group I (Rainey *et al.*, 1992; Stead, 1992). Clearly, it can be anticipated that an *oprF*-based comparative analysis of the members from the rRNA homology group I of *Pseudomonas*, including the numerous *P. syringae* pathovars, will provide an improved insight into their phylogenetic relatedness.

ACKNOWLEDGEMENTS

The authors wish to thank H. Joos, B. Lambert, F. Leyns (Plant Genetic Systems, Gent, Belgium) and D. Weller (USDA, Pullman, USA) for providing strains, and P. Marijnen (Center of Human Genetics, this University) for supplying the oligos. We are grateful to J. Mast (Laboratory for Physiology of Domestic Animals, Catholic University of Leuven) for help with the flow cytometry experiments. P.D. and R.D.M. are Senior Research Associates with the National Fund for Scientific Research (Belgium). This research was supported by grants from the National Fund for Scientific Research, from the Research Council of the Catholic University of Leuven (OT/90/24), and from the European Community (BIOTECH PL920196).

REFERENCES

- Arnheim, N. & Erlich, H. (1992). Polymerase chain reaction strategy. *Annu Rev Biochem* **61**, 131–156.
- Bellido, F., Martin, N. L., Siehnell, R. J. & Hancock, R. E. W. (1992). Reevaluation, using intact cells, of the exclusion limit and role of porin OprF in *Pseudomonas aeruginosa* outer membrane permeability. *J Bacteriol* **174**, 5196–5203.
- Butt, N. J., Virji, M., Vayreda, F., Lambden, P. R. & Heckels, J. E. (1990). Gonococcal outer-membrane protein PIB: comparative sequence analysis and localization of epitopes which are recognized by type-specific and cross-reacting monoclonal antibodies. *J Gen Microbiol* **136**, 2165–2172.
- Byström, A. S., Hjalmarsson, K. J., Wikström, P. M. & Björk, G. R. (1983). The nucleotide sequence of an *Escherichia coli* operon containing genes for the tRNA (m¹G) methyltransferase, the ribosomal proteins S16 and L19 and a 21-K polypeptide. *EMBO J* **2**, 899–905.
- Charbit, A., Ronco, J., Michel, V., Werts, C. & Hofnung, M. (1991). Permissive sites and topology of an outer membrane protein with a reporter epitope. *J Bacteriol* **173**, 262–275.
- Chen, R. & Henning, U. (1987). Nucleotide sequence of the gene for the peptidoglycan-associated lipoprotein of *Escherichia coli* K12. *Eur J Biochem* **163**, 73–77.
- Dean, D., Schachter, J., Dawson, C. R. & Stephens, R. S. (1992). Comparison of the major outer membrane protein variant sequence regions of B/Ba isolates: a molecular epidemiologic approach to *Chlamydia trachomatis* infections. *J Infect Dis* **166**, 383–392.
- Deich, R. A., Metcalf, B. J., Finn, C. W., Farley, J. E. & Green, B. A. (1988). Cloning of genes encoding a 15000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15000-dalton protein from *Haemophilus influenzae*. *J Bacteriol* **170**, 489–498.
- De Mot, R. & Vanderleyden, J. (1989). Application of two-dimensional protein analysis for strain fingerprinting and mutant analysis of *Azospirillum* species. *Can J Microbiol* **35**, 960–967.
- De Mot, R. & Vanderleyden, J. (1991). Purification of a root-adhesive outer membrane protein of root-colonizing *Pseudomonas fluorescens*. *FEMS Microbiol Lett* **81**, 323–328.
- De Mot, R., Proost, P., Van Damme, J. & Vanderleyden, J. (1992). Homology of the root adhesin of *Pseudomonas fluorescens* OE 28.3 with porin F of *P. aeruginosa* and *P. syringae*. *Mol & Gen Genet* **231**, 489–493.
- De Mot, R., Laeremans, T., Schoofs, G. & Vanderleyden, J. (1993). Characterization of the *recA* gene from *Pseudomonas fluorescens* OE 28.3 and construction of a *recA* mutant. *J Gen Microbiol* **139**, 49–57.
- Duchêne, M., Schweizer, A., Lottspeich, F., Krauss, G., Marget, M., Vogel, K., von Specht, B.-U. & Domdey, H. (1988). Sequence and transcriptional start site of the *Pseudomonas aeruginosa* outer membrane porin protein F gene. *J Bacteriol* **170**, 155–162.
- Engleberg, N. C., Howe, D. C., Rogers, J. E., Arroyo, J. & Eisenstein, B. I. (1991). Characterization of a *Legionella pneumophila* gene encoding a lipoprotein antigen. *Mol Microbiol* **5**, 2021–2029.
- Finnen, R. L., Martin, N. L., Siehnell, R. J., Woodruff, W. A., Rosok, M. & Hancock, R. E. W. (1992). Analysis of the *Pseudomonas aeruginosa* major outer membrane protein OprF by use of truncated OprF derivatives and monoclonal antibodies. *J Bacteriol* **174**, 4977–4985.
- Gentry-Weeks, C. R., Hultsch, A.-L., Kelly, S. M., Keith, J. M. & Curtiss, R., III (1992). Cloning and sequencing of a gene encoding a 21-kilodalton outer membrane protein from *Bordetella avium* and expression of the gene in *Salmonella typhimurium*. *J Bacteriol* **174**, 7729–7742.
- Georgiou, G., Poetschke, H. L., Stathopoulos, C. & Francisco, J. A. (1993). Practical applications of engineering Gram-negative bacterial surfaces. *Trends Biotechnol* **11**, 6–10.
- Goor, M., Vantomme, R., Swings, J., Gillis, M., Kersters, K. & De Ley, J. (1986). Phenotypic and genotypic diversity of *Pseudomonas tolaasii* and white line reacting organisms isolated from cultivated mushrooms. *J Gen Microbiol* **132**, 2249–2264.
- Gotoh, N., Wakebe, H. & Nishino, T. (1989a). Ultrastructural aspects of fragility of *Pseudomonas aeruginosa* outer membrane devoid of protein F. *FEMS Microbiol Lett* **59**, 51–54.
- Gotoh, N., Wakebe, H., Yoshihara, E., Nakae, T. & Nishino, T. (1989b). Role of protein F in maintaining structural integrity of the *Pseudomonas aeruginosa* outer membrane. *J Bacteriol* **171**, 983–990.
- Gotschlich, E. C., Seiff, M. & Blake, M. S. (1987). The DNA

- sequence of the structural gene of gonococcal protein III and the flanking region containing a repetitive sequence. Homology of protein III with enterobacterial OmpA proteins. *J Exp Med* **165**, 471–482.
- Harlowe, E. & Lane, D. (1988). *Antibodies. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Hayes, L. J., Pickett, M. A., Conlan, J. W., Ferris, S., Everson, J. S., Ward, M. E. & Clarke, I. N. (1990). The major outer-membrane proteins of *Chlamydia trachomatis* serovars A and B: intra-serovar amino acid changes do not alter specificities of serovar- and C subspecies-reactive antibody-binding domains. *J Gen Microbiol* **136**, 1559–1566.
- Higgins, D. G. & Sharp, P. M. (1988). CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237–244.
- Hughes, E. E., Gilleland, L. B. & Gilleland, H. E., Jr (1992). Synthetic peptides representing epitopes of outer membrane protein F of *Pseudomonas aeruginosa* that elicit antibodies reactive with whole cells of heterologous immunotype strains of *P. aeruginosa*. *Infect Immun* **60**, 3497–3503.
- Kaltenboeck, B., Kousoulas, K. G. & Storz, J. (1993). Structures and allelic diversity and relationships among the major outer membrane protein (*ompA*) genes of the four chlamydial species. *J Bacteriol* **175**, 487–502.
- Klose, M., MacIntyre, S., Schwarz, H. & Henning, U. (1988a). The influence of amino substitutions within the mature part of an *Escherichia coli* outer membrane protein (OmpA) on assembly of the polypeptide into its membrane. *J Biol Chem* **263**, 13297–13302.
- Klose, M., Schwarz, H., MacIntyre, S., Freudl, R., Eschbach, M.-L. & Henning, U. (1988b). Internal deletions in the gene for an *Escherichia coli* outer membrane protein define an area possibly important for recognition of the outer membrane by this polypeptide. *J Biol Chem* **263**, 13291–13296.
- Klugman, K. P., Gotschlich, E. C. & Blake, M. S. (1989). Sequence of the structural gene (*rmpM*) for the class 4 outer membrane protein of *Neisseria meningitidis*, homology of the protein to gonococcal protein III and *Escherichia coli* OmpA, and construction of meningococcal strains that lack class 4 protein. *Infect Immun* **57**, 2066–2071.
- Lampe, M. F., Suchland, R. J. & Stamm, W. E. (1993). Nucleotide sequence of the variable domains within the major outer membrane protein gene from serovariants of *Chlamydia trachomatis*. *Infect Immun* **61**, 213–219.
- Lawrence, J. G., Ochman, H. & Hartl, D. L. (1991). Molecular and evolutionary relationships among enteric bacteria. *J Gen Microbiol* **137**, 1911–1921.
- Little, M., Fuchs, P., Breitling, F. & Dübel, S. (1993). Bacterial surface presentation of proteins and peptides: an alternative to phage technology. *Trends Biotechnol* **11**, 3–5.
- Lugtenberg, B. J. J., de Weger, L. A. & Bennett, J. W. (1991). Microbial stimulation of plant growth and protection from disease. *Curr Opin Biotechnol* **2**, 457–464.
- Maiden, M. C. J., Suker, J., McKenna, A. J., Bygraves, J. A. & Feavers, I. M. (1991). Comparison of the class 1 outer membrane proteins of eight serological reference strains of *Neisseria meningitidis*. *Mol Microbiol* **5**, 727–736.
- Matsudaira, P. T. (1989). *A Practical Guide to Protein and Peptide Purification for Microsequencing*. San Diego: Academic Press.
- Mazzola, M., Cook, R. J., Tomashow, L. S., Weller, D. M. & Pierson, L. S., III (1992). Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent *Pseudomonas* in soil habitats. *Appl Environ Microbiol* **58**, 2616–2624.
- Morona, R., Klose, M. & Henning, U. (1984). *Escherichia coli* K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. *J Bacteriol* **159**, 570–578.
- Munson, R. S., Jr, Grass, S. & West, R. (1993). Molecular cloning and sequence of the gene for outer membrane protein P5 of *Haemophilus influenzae*. *Infect Immun* **61**, 4017–4020.
- Murphy, T. F., Kirkham, C. & Lesse, A. J. (1993). The major heat-modifiable outer membrane protein CD is highly conserved among strains of *Branhamella catarrhalis*. *Mol Microbiol* **10**, 87–97.
- Mutharia, L. M. & Hancock, R. E. W. (1985). Characterization of two surface-localized antigenic sites on porin protein F of *Pseudomonas aeruginosa*. *Can J Microbiol* **31**, 381–386.
- Nelson, M. B., Apicella, M. A., Murphy, T. F., Vankeulen, H., Spotila, L. D. & Rekosh, D. (1988). Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. *Infect Immun* **56**, 128–134.
- Nikaido, H., Nikaido, K. & Harayama, S. (1991). Identification and characterization of porins in *Pseudomonas aeruginosa*. *J Biol Chem* **266**, 770–779.
- O'Sullivan, D. J. & O'Gara, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol Rev* **50**, 662–676.
- Rainey, P. B., Brodey, C. L. & Johnstone, K. (1992). Biology of *Pseudomonas tolaasii*, cause of brown blotch disease of the cultivated mushroom. *Adv Plant Pathol* **8**, 95–117.
- Srikumar, R., Chin, A. C., Vachon, V., Richardson, C. D., Ratcliffe, M. J. H., Saarinen, L., Käyhty, H., Mäkelä, P. H. & Coulton, J. W. (1992). Monoclonal antibodies specific to porin of *Haemophilus influenzae* type b: localization of their cognate epitopes and tests of their biological activities. *Mol Microbiol* **6**, 665–676.
- Stead, D. E. (1992). Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. *Int J Syst Bacteriol* **42**, 281–295.
- Sugawara, E. & Nikaido, H. (1992). Pore-forming activity of OmpA protein of *Escherichia coli*. *J Biol Chem* **267**, 2507–2511.
- Surrey, T. & Jähnig, F. (1992). Refolding and oriented insertion of a membrane protein into a lipid bilayer. *Proc Natl Acad Sci USA* **89**, 7457–7461.
- Szwajcer-Dey, E., Rasmussen, J., Meldal, M. & Breddam, K. (1992). Proline-specific endopeptidases from microbial sources: isolation of an enzyme from a *Xanthomonas* sp. *J Bacteriol* **174**, 2454–2459.
- Ullstrom, C. A., Siehnell, R., Woodruff, W., Steinbach, S. & Hancock, R. E. W. (1991). Conservation of the gene for outer membrane protein OprF in the family *Pseudomonadaceae*: sequence of the *Pseudomonas syringae* *oprF* gene. *J Bacteriol* **173**, 768–775.
- Vlassak, K., Van Holm, L., Duchateau, L., Vanderleyden, J. & De Mot, R. (1992). Isolation and characterization of fluorescent *Pseudomonas* associated with the roots of rice and banana grown in Sri Lanka. *Plant Soil* **145**, 51–63.
- Vogel, H. & Jähnig, F. (1986). Models for the structure of outer-membrane proteins of *Escherichia coli* derived from Raman spectroscopy and prediction methods. *J Mol Biol* **190**, 191–199.
- Weller, D. M. & Cook, R. J. (1983). Suppression of take-all of wheat by seed treatments with fluorescent *Pseudomonads*. *Phytopathology* **73**, 463–469.
- Won, J. & Griffith, R. W. (1993). Cloning and sequencing of the gene encoding a 31-kilodalton antigen of *Haemophilus somnus*. *Infect Immun* **61**, 2813–2821.
- Wong, R. S. Y., Jost, H. & Hancock, R. E. W. (1993). Linker-

insertion mutagenesis of *Pseudomonas aeruginosa* outer membrane protein OprF. *Mol Microbiol* **10**, 283–292.

Wong, W. C. & Preece, T. F. (1979). Identification of *Pseudomonas tolaasii*: the white line in agar and mushroom tissue block rapid pitting tests. *J Appl Bacteriol* **47**, 401–407.

Woodruff, W. A. & Hancock, R. E. W. (1989). *Pseudomonas aeruginosa*

outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. *J Bacteriol* **171**, 3304–3309.

Received 16 September 1993; revised 6 December 1993; accepted 10 January 1994.