A phosphate-starvation-inducible outer-membrane protein of *Pseudomonas fluorescens* Ag1 as an immunological phosphate-starvation marker

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A phosphate-starvation-inducible outer-membrane protein of *Pseudomonas fluorescens* Ag1, expressed at phosphate concentrations below 0.08–0.13 mM, was purified and characterized. The purification method involved separation of outer-membrane proteins by SDS-PAGE and extraction of the protein from nitrocellulose or PVDF membranes after electrotransfer of proteins to the membranes. The N-terminal amino acid sequence of the purified protein, called Psi1, did not show homology to any known proteins, and in contrast to the phosphate-specific porin OprP of *P. aeruginosa* its mobility in SDS-PAGE was not affected by solubilization temperature. An antiserum against Psi1 recognized a protein of *M*_r 55 000 in four other *P. fluorescens* strains among 24 tested strains representing *Pseudomonas* rRNA homology group I, showing antigenic heterogeneity within this group. A method for immunofluorescence microscopy involving cell permeabilization was adapted to visualize cell-specific expression of Psi1 in *P. fluorescens* exposed to limiting amounts of phosphate. This approach should be useful for further exploration of Psi1 as a marker to study the availability of phosphate to *P. fluorescens* in natural environments.

Keywords: phosphate-starvation protein, *Pseudomonas fluorescens*, immunochemical single-cell detection

INTRODUCTION

The fluorescent pseudomonads are a group of bacteria which have received special attention due to the possible biotechnological exploitation of strains that promote plant growth (Keel *et al.*, 1990; Höfte *et al.*, 1991; Mazzola & Cook, 1991) and of strains able to degrade xenobiotic compounds (Ramos *et al.*, 1994). The utility of beneficial *Pseudomonas* strains requires successful colonization of natural environments but knowledge about the availability of substrates and nutrients in these habitats is scarce. For example, phosphorus is probably

not easily available to micro-organisms in the soil (Jungk *et al.*, 1993; De Weger *et al.*, 1994). As lack of phosphate severely reduces survival of pseudomonads in monoculture starvation experiments (Kragelund & Nybroe, 1994; Eberl *et al.*, 1996) phosphate limitation may represent a real and severe stress in natural environments.

When fluorescent pseudomonads encounter phosphate limitation they express a set of phosphate-starvation genes (Givskov *et al.*, 1994; Kragelund *et al.*, 1995). Lack of phosphate in complex, natural environments might therefore be monitored by reporter systems for gene expression rather than by chemical analyses that provide little information on the bioavailability of phosphate. Hence, transcriptional fusions between phosphate-regulated promoters and *lacZ* (De Weger *et al.*, 1994) or *luxAB* (Kragelund & Nybroe, 1995) have been used to determine phosphate availability in plant–soil ecosystems. However, *lacZ*-tagged cells typically cannot be

Abbreviations: DAPI, 4',6-diamidino-2-phenylindoline; DMM, Davis minimal medium; DMM-P, Davis minimal medium depleted of phosphate; NCBI, National Center for Biotechnology Information; OM, outer membrane; Psi1, phosphate-starvation-inducible protein 1.

The PIR data library accession number for the sequence reported in this paper is P80694.

identified reliably at the single-cell level and furthermore a background of β -galactosidase activity may be present in the environment. In parallel, expression of bioluminescence from *lux*-tagged cells requires active metabolism and a sensitive charge-coupled device camera is needed for single-cell detection. Alternatively, the use of a starvation protein as an immunological marker allows detection of single cells by immunofluorescence microscopy provided that the selected marker is abundantly expressed. In addition, an immunological marker system can target cells which have not been genetically modified by insertion of a reporter gene.

The fluorescent pseudomonad *Pseudomonas aeruginosa* expresses abundant outer-membrane proteins as a response to phosphate limitation: the phosphate-specific porin OprP (Hancock *et al.*, 1982) and the polyphosphate-specific porin OprO (Hancock *et al.*, 1992). Phosphate-starvation-induced outer-membrane proteins have also been observed in some, but not all, *P. fluorescens* strains investigated (Poole & Hancock, 1986; Poole *et al.*, 1987; Kragelund & Nybroe, 1994) and these outer-membrane proteins represent potential immunological markers. The purpose of this work was to develop an immunological marker system for phosphate-starved *P. fluorescens*. We have purified a phosphate-starvation-induced outer-membrane protein of *P. fluorescens* strain Ag1 and characterized the protein by amino acid analysis and N-terminal amino acid sequence analysis. An antibody against the phosphate-starvation-induced protein has been raised and used to detect individual cells of *P. fluorescens* depleted of phosphate. The perspective of this approach is to use the antibody to study the availability of phosphate for pseudomonads in both simple systems and complex natural environments.

METHODS

Bacterial strains and culture media. *Pseudomonas fluorescens* strain Ag1 was originally isolated from soil. Specificity of the antibody produced during this study was tested against a panel of environmental *P. fluorescens* and *P. putida* strains and against type strains of rRNA homology group I pseudomonads (Table 1). Under non-starved conditions, the bacteria were grown aerobically at room temperature (23–25 °C) in Davis minimal medium, pH 7.3 [DMM; 0.4% glucose, 30 mM K_2HPO_4 , 14 mM KH_2PO_4 , 0.4 mM $MgSO_4$, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate supplemented with 1 ml trace element solution l^{-1}]. The trace element solution contained 20 mg $CoCl_2 \cdot 6H_2O$, 30 mg H_3BO_3 , 10 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $CuCl_2 \cdot 2H_2O$, 2 mg $NiCl_2 \cdot 6H_2O$, 3 mg $NaMoO_4 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, and 2.6 mg $MnSO_4 \cdot H_2O$ dissolved in 1 l demineralized water.

Phosphate starvation. Prior to starvation experiments, cells were grown overnight aerobically in DMM. The overnight culture was diluted to an OD_{600} of 0.1 as measured by a Perkin Elmer Lambda Bio Spectrometer with a 1 cm light path. When the cultures were again growing exponentially (OD_{600} 0.4–0.6), the cells were harvested by centrifugation at 6000 g for 10 min at room temperature, and washed twice with DMM without phosphate but supplemented with 10 mM HEPES, pH 7.3, to maintain the pH (DMM-P). The cells were

then resuspended at the original cell density in DMM-P. After 15–25 h, the cells were harvested by centrifugation at 6000 g for 10 min.

Induction studies. *P. fluorescens* Ag1 was grown overnight aerobically in DMM supplemented with 0.8% KNO_3 and 0.2% Casamino acids (Difco) to reduce cell aggregation. A series of cultures with an OD_{600} of 0.1 was prepared by diluting the overnight culture. When the cultures were again growing exponentially (OD_{600} 0.4–0.6), the cells were harvested by centrifugation at 6000 g for 10 min, washed twice with DMM-P supplemented with 0.04, 0.08, 0.13, 0.17, 0.22, 0.25, 0.30, 0.35, 0.40, 0.45 or 0.50 mM inorganic phosphate, and resuspended in the respective medium at an OD_{600} of 0.1. After incubation for 2 h 20 min the protein synthesis was stopped by adding 200 p.p.m. chloramphenicol. Cells were harvested by centrifugation at 10000 g for 10 min at 4 °C. The cell pellets were analysed by immunoblotting (see below).

The concentrations of inorganic phosphate in the supernatant at the beginning and at the end of the experiment were determined colorimetrically by a modified method of Heinonen & Lahti (1981). Reaction solution (1 ml) containing 1 vol. 10 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 vol. 2.5 mM H_2SO_4 , and 2 vols acetone, was added to 200 μ l sample (containing 2.5–200 nmol phosphate). A_{340} was measured.

SDS-PAGE. Protein composition was analysed by SDS-PAGE and silver staining (Blum *et al.*, 1987). SDS-PAGE was performed in 10% (w/v) acrylamide and 0.27% (w/v) N,N' -methylenebisacrylamide separation gels overlaid with 4.5% acrylamide/0.12% methylenebisacrylamide stacking gels. Samples were, if not otherwise stated, solubilized by boiling for 5 min in sample buffer (62.5 mM Tris/HCl, pH 6.8, containing 2.0% SDS, 6.25% glycerol, 0.005% bromophenol blue) including 0.47% DTT (reduced samples) or without DTT (non-reduced samples).

Cell fractionation. The procedure for cell fractionation was modified from Yamamoto *et al.* (1987). Phosphate-starved cells of *P. fluorescens* Ag1 were harvested by centrifugation at 7000 g for 10 min at room temperature. The supernatant, representing the culture medium, was filtered through 0.2 μ m pore-size polycarbonate filters (Nuclepore). The cells were subsequently washed and incubated for 10 min at room temperature in 10 mM Tris/HCl, pH 7.5, 25% sucrose. After centrifugation as above, the procedure was repeated including 1 mM EDTA in the buffer. The supernatants, representing extracellular material, were collected. The cells were then resuspended in ice-cold water and shaken vigorously for 10 min on ice. Following centrifugation at 9000 g for 10 min at 4 °C the supernatant, representing the periplasmic fraction, was removed. The cell pellet was resuspended in 10 mM Tris/HCl and sonicated for 2 min on ice using an MSE 150W Ultrasonic disintegrator (Sanoy), probe diameter 9.5 mm, at 10 μ m amplitude. Remaining whole cells were removed by centrifugation at 9000 g for 10 min and cell walls were subsequently pelleted by centrifugation at 47000 g for 2 h at 4 °C. The new supernatant represented the cytoplasmic fraction. Half of the pellet was immediately frozen (cell wall fraction) while the remaining half was used to prepare outer membranes (see below).

Protein concentrations were, throughout this work, determined by the bicinchoninic acid protein assay (Pierce) using BSA as a standard.

Preparation of outer membranes. Outer membranes were isolated by the Sarkosyl method (Filip *et al.*, 1973), essentially as described by Kragelund & Nybroe (1994). However, when

Table 1. Specificity of the antibody raised against Psi1 of *P. fluorescens* Ag1 tested by immunoblotting analysis of strains from the genus *Pseudomonas* and of *Escherichia coli*

Each sample contained approximately 10^7 cells; all samples were non-reduced.

Strain	Biovar	Strength of reaction* of antibody with protein from cells which were:	
		Solubilized at 100 °C prior to electrophoresis	Solubilized at room temperature prior to electrophoresis
<i>P. fluorescens</i> Ag1	II	+++ (55)	+ (55) ++ (70–110)
<i>P. fluorescens</i> ON24†	I	—	—
<i>P. fluorescens</i> MM5†	I	—	—
<i>P. fluorescens</i> MM6†	II	—	—
<i>P. fluorescens</i> DF8†	II	—	—
<i>P. fluorescens</i> DF57†	II	—	—
<i>P. fluorescens</i> DF61†	III	—	—
<i>P. fluorescens</i> DF23†	IV	—	—
<i>P. fluorescens</i> DF18†	IV	—	—
<i>P. fluorescens</i> ON13†	V	++ (55)	+ (55)
<i>P. fluorescens</i> ON25†	V	++ (55)	++ (55)
<i>P. fluorescens</i> DF17†	V or VI‡	—	—
<i>P. fluorescens</i> ON5†	V or VI‡	—	—
<i>P. fluorescens</i> DSM 50090 ^T	I	—	—
<i>P. fluorescens</i> DSM 50106	II	+++ (55)	+++ (55)
<i>P. fluorescens</i> DSM 50124	III	—	—
<i>P. fluorescens</i> DSM 50415	IV	—	—
<i>P. fluorescens</i> DSM 50148	V	+++ (55)	+ (55)
<i>P. putida</i> DSM 291 ^T	A	—	—
<i>P. putida</i> DSM 50222	B	—	—
<i>P. stutzeri</i> DSM 5190 ^T	—	—	—
<i>P. aeruginosa</i> DSM 50071 ^T	—	—	—
<i>P. chlororaphis</i> DSM 50083 ^T	—	—	—
<i>P. syringae</i> ATCC 19310 ^T	—	—	—
<i>E. coli</i> DSM 498	—	—	—

* + + +, Strong reaction; + +, medium reaction; +, weak reaction; —, no reaction. Figures in parentheses are $10^{-3} \times M_r$ of the reactive protein.

† Classified by the API 20NE system supplemented by additional tests (Sørensen *et al.*, 1992).

‡ New biotype VI according to Barrett *et al.* (1982).

outer membranes were prepared from whole phosphate-starved cells, sonication with an MSE 150W Ultrasonic Disintegrator (settings as above) for 10×2 min on ice was required to rupture cells. The membrane preparations were stored at -20 °C after addition of 100 U aprotinin ml^{-1} (Sigma).

Purification of protein from nitrocellulose membranes. Outer-membrane proteins of phosphate-starved *P. fluorescens* Ag1 were separated by SDS-PAGE under non-reducing conditions as described above. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell BA85, $0.45 \mu\text{m}$) by semi-dry electroblotting (Kem-En-Tec) at 0.8 mA cm^{-2} for 2 h. To purify the protein of interest, its position on the nitrocellulose membrane was identified by staining part of the membrane with 0.2% Ponceau S in 5% acetic acid (Sigma). The protein was cut out from the unstained part of the nitrocellulose membrane using the stained part as a template

and purified by elution with formic acid (Judd, 1988). Purity of the eluted protein was monitored by SDS-PAGE and protein-specific silver staining (Blum *et al.*, 1987) or lipopolysaccharide-specific periodate/silver staining (Tsai & Frasch, 1982). The preparation was kept at -20 °C if not used immediately.

Amino acid analysis and N-terminal amino acid sequence analysis. Outer membranes were subjected to SDS-PAGE as above followed by electroblotting onto a PVDF membrane (Bio-Rad) (Matsudaira, 1987). The protein of interest was localized by staining with Coomassie R250 (Merck) and cut out. The stained band was thoroughly washed in demineralized water, dried under nitrogen and used for amino acid analysis and N-terminal amino acid sequencing. The protein was hydrolysed at 110 °C in 6 M HCl, 0.05% phenol and $5 \mu\text{l}$ 1% 3,3'-dithiodipropionic acid for 24 h and 72 h (Barkholt & Jensen, 1989). Cysteine/cystine was derivatized with 3,3'-

dithiodipropionic acid during hydrolysis. Amino acids were determined by cation-exchange chromatography and post-column derivatization with *o*-phthalaldehyde. N-terminal amino acid sequence analysis of PVDF-bound protein was carried out by automated Edman degradation using an Applied Biosystems Procise 494 sequencer as described by the manufacturer. Multiple alignments of protein sequences and nucleotide sequences were performed using the BLAST network service at the National Center for Biotechnology Information.

Production of antiserum. Antiserum against purified protein was made by immunizing two mice (strain Ssc:CF1) subcutaneously each with approximately 5 µg purified protein mixed (1:1) with Freund's incomplete adjuvant (State Serum Institute, Denmark) four times at 2 week intervals. Immunization was done by the State Serum Institute, Denmark. Two weeks after the fourth immunization the mice were killed and blood was collected. Serum was obtained by centrifugation and NaN₃ (15 mM) was added as a preservative.

Immunoblotting. SDS-PAGE and electrotransfer of protein to nitrocellulose membranes were as described above. After electrotransfer, the blot was blocked for 20 min in 50 mM Tris/HCl, pH 10.2, 150 mM NaCl, 2% Tween 20, 0.5% BSA, 1% casein. After blocking the nitrocellulose membrane was incubated overnight at room temperature with antiserum. The antiserum specific to the phosphate-starvation protein was diluted 1:30000 and antiserum against OprF (Kragelund *et al.*, 1996) was diluted 1:17500 with 50 mM Tris/HCl, pH 10.2, 150 mM NaCl, 0.05% Tween 20, 0.5% BSA, 0.3% casein. After washing three times in washing buffer (50 mM Tris/HCl, pH 10.2, 150 mM NaCl, 0.05% Tween 20, 0.05% BSA), the nitrocellulose membrane was incubated for 2 h with alkaline-phosphatase-conjugated rabbit anti-mouse immunoglobulins (1:1000 in dilution buffer) (DAKO). After three washes, colour was developed with 0.1 mg nitroblue tetrazolium ml⁻¹ (Sigma), 0.06 mg 5-bromo-4-chloro-3-indolyl phosphate ml⁻¹ (Sigma), 20 mM MgCl₂ in 100 mM ethanolamine, pH 9.6.

Dot-immunoblotting. Samples were collected on a nitrocellulose membrane by filtration. The membrane was immunostained essentially as described for immunoblotting. However incubation with PBS (25 mM sodium phosphate, pH 7.4, 125 mM NaCl) adjusted to pH 2.8 was included to inhibit indigenous alkaline phosphatase activity (Olsen & Rice, 1989). The filters were treated with 100 mM Tris/HCl, pH 7.5, 50 mM sodium metaperiodate for 3 × 20 min at room temperature before incubation with antibodies.

Immunofluorescence microscopy. The procedure for immunofluorescence microscopy was modified from Maddock & Shapiro (1993) and Harry *et al.* (1995). After vortexing to disperse cell aggregates, cells were fixed by adding buffered formaldehyde to a final concentration of 2%, and kept on ice for 1 h and at room temperature for at least 3 h. All subsequent steps were performed at room temperature if not otherwise stated. Fixed cells were washed three times in PBS, submitted to oxidation of carbohydrates by 1% sodium metaperiodate for 15 min, washed once in PBS and quenched for 15 min in 50 mM NH₄Cl. Following a final wash in demineralized water, samples of 50 µl were transferred to poly-L-lysine-coated slides. After 30 s the liquid was removed and the slides allowed to dry. The cells were then permeabilized by transfer to -20 °C methanol for 5 min and -20 °C acetone for 30 s and again allowed to dry. The slides were immunostained as described for immunoblotting, with the following modifications: all buffers were at pH 7.6, the phosphate starvation protein antiserum was diluted 1:100, and the secondary

antibody was fluorescein-conjugated goat anti-mouse immunoglobulin (DAKO) diluted 1:20.

RESULTS

Purification of Psi1

Outer-membrane preparations from exponentially growing and from phosphate-starved *P. fluorescens* Ag1 were analysed by SDS-PAGE. A protein of *M_r* 55000, which we called phosphate-starvation-inducible protein 1 (Psi1), appeared under phosphate starvation (Fig. 1, compare lanes 1 and 2) but not under either carbon or nitrogen starvation (data not shown). When reduced, Psi1 appeared to have a slightly higher *M_r*, approximately 56000, than non-reduced Psi1 (Fig. 1, lanes 2 and 4). When outer-membrane samples were solubilized in sample buffer at room temperature instead of at 100 °C, the mobility of the Psi1 protein was unaltered, but the Psi1 protein band was less intense.

The *M_r* 55000 form of Psi1 was purified from outer membranes by elution from nitrocellulose membranes after electrotransfer from SDS-polyacrylamide gels. The protein preparation obtained was electrophoretically pure as demonstrated by SDS-PAGE and protein-specific silver staining (Fig. 2, lane 3). No lipopolysaccharide could be detected by carbohydrate-specific periodate/silver staining (data not shown). The purified Psi1 protein migrated at *M_r* 56000, whether heated or not prior to electrophoresis (Fig. 2, lanes 3 and 4). The *M_r* 56000 form probably represents fully denatured protein obtained either by treatment with SDS plus reducing agent or by the acid treatment included in the purification procedure. The recovery of Psi1 was approximately 40 µg per 1000 µg outer-membrane-protein prep-

Fig. 1. SDS-PAGE of outer-membrane proteins of *P. fluorescens* Ag1 starved of phosphate. Lanes with odd numbers, outer membranes from exponential phase cells; lanes with even numbers, outer membranes from phosphate-starved cells. Samples in lanes 1 and 2 are non-reduced and samples in lanes 3 and 4 are reduced. All samples were solubilized in sample buffer at 100 °C for 5 min prior to electrophoresis. Gels are silver stained. The *M_r* 55000 and the 56000 forms of Psi1 are marked by arrows. The migration positions of *M_r* standards are indicated.

Fig. 2. SDS-PAGE of purified Psi1 protein. Lane 1, outer membranes from exponential phase *P. fluorescens* Ag1 cells; lane 2, outer membranes from phosphate-starved *P. fluorescens* Ag1 cells; lane 3, purified Psi1, solubilized at 100 °C prior to electrophoresis; lane 4, purified Psi1, solubilized at room temperature prior to electrophoresis. All samples are non-reduced. Gels are silver stained. The migration positions of M_r standards are indicated.

aration, indicating that it is a major component of the outer membrane of starving cells.

Amino acid composition and N-terminal amino acid sequence analysis

To determine whether Psi1 was related to other known outer-membrane proteins, amino acid analysis and N-terminal amino acid sequence analysis of Psi1 were performed. The analyses were done on PVFD-membrane-bound purified protein. The amino acid composition of Psi1 is listed in Table 2 along with the previously published amino acid compositions of the phosphate-starvation-induced outer-membrane porins OprP of *P. aeruginosa* and PhoE of *Escherichia coli* and of the major outer-membrane protein OprF of *P. fluorescens* and *P. aeruginosa*. The overall amino acid composition appeared fairly similar between the compared proteins, containing 34–38% charged residues (Asp, Glu, His, Lys and Arg) and 31–38% hydrophobic residues (Pro, Ala, Val, Met, Ile, Leu, Phe and Trp). The phosphate-starvation-induced proteins (Psi1, OprP and PhoE) showed lower contents of proline and valine than OprF. Psi1 furthermore differed from OprP in a higher Glu/Gln ratio and a lower Gly content. The sequence of the 39 N-terminal amino acids of Psi1 was: AVDAKLLDMLKANGQISASQYTELQAELAKDQKEKQIAQ. A homology search was carried out with all available sequence databases but did not reveal any homology to known proteins.

Specificity of the polyclonal antiserum raised against Psi1

A polyclonal antiserum against purified Psi1 was raised in mice and the specificity of this antiserum was determined by immunoblot analysis of outer-membrane

Table 2. Amino acid composition of the phosphate-starvation-induced outer-membrane protein of *P. fluorescens* Ag1, Psi1, and amino acid composition of selected outer-membrane proteins of *P. fluorescens*, *P. aeruginosa* and *E. coli*

Amino acid	Composition (mol%)				
	<i>P. fluorescens</i>		<i>P. aeruginosa</i>		<i>E. coli</i>
	Psi1*	OprF†	OprP‡	OprF§	PhoE
Asp/Asn	14.5	13.2	14.8	15.3	17.6
Thr	6.3	4.6	6.8	5.4	7.0
Ser	6.7	6.3	8.3	6.4	5.4
Glu/Gln	11.6	9.9	8.0	10.2	9.1
Pro	2.0	5.3	1.6	3.1	0.9
Gly	11.4	10.9	14.3	11.2	10.9
Ala	10.0	12.3	10.4	9.5	8.1
Cys	0.1	0.0	0.0	1.3	0.0
Val	4.9	9.6	5.5	8.5	3.6
Met	0.9	0.3	1.3	1.7	2.1
Ile	3.9	2.0	1.8	2.4	3.9
Leu	8.2	5.0	6.8	4.8	6.4
Tyr	4.0	5.0	5.2	5.4	6.7
Phe	3.6	3.3	3.6	3.7	6.4
His	1.7	2.0	0.8	1.8	0.3
Lys	6.3	5.0	4.9	4.3	7.0
Arg	4.1	5.0	5.7	4.2	3.6
Trp	ND	0.3	ND	0.7	0.9

ND, Not determined.

* Values were obtained after 24 h hydrolysis, except for Val and Ile (72 h hydrolysis). Values for Thr and Ser were obtained by extrapolation to zero time of hydrolysis. Cys was determined as half-cystine.

† Derived from the nucleotide sequence of the protein (De Mot *et al.*, 1994).

‡ Amino acid analysis (Worebec *et al.*, 1988). Values were obtained after 24 h hydrolysis.

§ Amino acid analysis (Mizuno & Kageyama, 1979). Means of values obtained at 24, 48, and 72 h hydrolysis. Values for Thr and Ser were obtained by extrapolation to zero time of hydrolysis. Cys was determined as half-cystine. The values for half-cystine and Met were obtained by amino acid analysis after performic acid oxidation of the samples.

|| Derived from the nucleotide sequence of the protein (Overbeeke *et al.*, 1983).

preparations from *P. fluorescens* Ag1. The antiserum recognized a protein of M_r 55000 in outer membranes from phosphate-starved cells, whereas no reaction with outer membranes from non-starved cells could be seen (Fig. 3, compare lanes 1 and 2). If the outer-membrane preparation was not heated prior to electrophoresis a smear of varying width, M_r approximately 70000–110000, appeared in addition to the M_r 55000 band (Fig. 3, lane 3). When tested against a number of *P. fluorescens* strains representing all biovars of this species and against a panel of type strains from RNA homology

Fig. 3. Specificity of the polyclonal antiserum raised against Psi1 tested by immunoblotting. Lanes with odd numbers, outer membranes from phosphate-starved *P. fluorescens* Ag1; lanes with even numbers, outer membranes from exponential-phase *P. fluorescens* Ag1. Samples in lanes 1 and 2 were solubilized at 100 °C prior to electrophoresis and samples in lanes 3 and 4 were solubilized at room temperature prior to electrophoresis. All samples are non-reduced.

Fig. 4. Cellular localization of Psi1 of *P. fluorescens* demonstrated by dot immunobinding analysis of subcellular fractions. (a) Incubation with antibody against Psi1; (b) incubation with antibody against OprF. Dot 1, culture medium; dot 2, extracellular material released by Tris/sucrose buffer; dot 3, extracellular material released by Tris/sucrose/EDTA buffer; dot 4, periplasmic fraction; dot 5, cytoplasmic fraction; dot 6, cell wall fraction; dot 7, outer membrane fraction. Each dot contains 5 µg total protein.

group I pseudomonads (Table 1) and *Escherichia coli*, the antiserum reacted with proteins with relative molecular masses of approximately 55000 in only the following strains of *P. fluorescens*: DSM 50106 (biovar II), DSM 50148 (biovar V), ON13 (biovar V) and ON25 (biovar V) (see Table 1), whether or not the cells were heated prior to electrophoresis.

Cellular localization

Cells of phosphate-starved *P. fluorescens* Ag1 were fractionated and the localization of Psi1 was determined by dot-immunobinding (Fig. 4). The localization of a well-known outer-membrane marker, OprF (Hancock *et al.*, 1990), was determined as a control. Psi1 and OprF co-localized to cell wall and outer-membrane fractions. Neither of the proteins was found in extracellular material, in the periplasm or in the cytoplasm.

The antibody raised against Psi1 did not react with intact phosphate-starved Ag1 cells in immunofluorescence microscopy, suggesting either that the protein did not contain surface-exposed epitopes or that

Fig. 5. Phosphate-starved *P. fluorescens* Ag1 detected by immunofluorescence microscopy using antiserum against Psi1 after fixation and permeabilization of cells. Bar, 5 µm.

Fig. 6. Induction of Psi1 examined by immunoblotting. Cells were grown at the following phosphate concentrations: 0.04 mM (lane 1), 0.08 mM (lane 2), 0.13 mM (lane 3), 0.17 mM (lane 4). The blot was co-incubated with antiserum against Psi1 and antibody against OprF. Lanes 5 and 6 are controls and represent phosphate-starved cells incubated with anti Psi1 and anti OprF (appearing as two bands), respectively. The location of Psi1 is shown by a solid arrow and that of OprF by open arrows.

such epitopes were masked. However, a distinct surface staining of starved cells was apparent when the cells were fixed with formaldehyde, permeabilized with methanol/acetone and treated with metaperiodate to oxidize cell wall carbohydrates prior to immunostaining (Fig. 5). Treatment with lysozyme to disrupt cell wall carbohydrates was less efficient than metaperiodate oxidation (data not shown). Exponentially growing cells submitted to the above-mentioned procedure gave no signal (data not shown).

Expression of Psi1 at different phosphate concentrations

The lowest phosphate concentration resulting in expression of Psi1 was determined by incubating Ag1 cells at phosphate concentrations ranging from 0 to 0.50 mM

and subsequently analysing the cells by immunoblotting to detect both Psi1 and the constitutive marker OprF. When cells were transferred to phosphate-free medium, Psi1 could be detected by immunoblotting after approximately 70 min. To ensure detection of Psi1, induction studies were performed for 2 h 20 min. During this period there was no detectable consumption of phosphate in the cultures. It was found that Psi1 was induced at phosphate concentrations between approximately 0.08 and 0.13 mM (Fig. 6).

DISCUSSION

Characterization of the phosphate-starvation-inducible protein Psi1

Under conditions of phosphate limitation fluorescent pseudomonads express a set of starvation proteins. For example, at least 56 phosphate-starvation proteins are induced in *P. putida* strain KT2442 (Givskov *et al.*, 1994), and in *P. fluorescens* strain DF57 induction of several phosphate-starvation genes has been reported (Kragelund *et al.*, 1995). In the present work we describe a major phosphate-starvation-inducible protein, Psi1, from *P. fluorescens* strain Ag1. The Psi1 protein from strain Ag1 is induced during phosphate deprivation and is likely to be a member of the phosphate-starvation (Pho) regulon (Siehnel *et al.*, 1990; Siehnel *et al.*, 1992) but the protein is not a general starvation marker. Psi1 is induced at phosphate concentrations below 0.08–0.13 mM. This limit is close to the limit reported for the phosphate-induced protein OprP from *P. aeruginosa* (0.10–0.20 mM; Hancock *et al.*, 1982) whereas De Weger *et al.* (1994) reported that a phosphate-regulated *lacZ* fusion in *P. fluorescens* strain WCS358 was induced when the phosphate concentration dropped below approximately 0.03 mM. This difference may be due to differences in experimental set-up: the threshold for the WCS358 gene fusion was determined while the cells were continuously depleting a medium rich in phosphate, while we used incubations in minimal medium at fixed phosphate concentrations. Finally, the sensitivity of the detection systems may differ.

Both cell fractionation experiments and immunocytochemical evidence demonstrate that the M_r 55 000 Psi1 protein is exclusively localized in the outer membrane. In *P. aeruginosa*, the M_r 48 000 phosphate-specific outer-membrane porin OprP is induced during limitation for phosphate, and outer-membrane proteins with comparable properties have been demonstrated in at least one *P. fluorescens* strain (Poole & Hancock, 1986; Poole *et al.*, 1987). We therefore addressed the possibility that Psi1 could represent a *P. fluorescens* homologue to OprP. However, the amino acid compositions of Psi1 and OprP were distinctly different and N-terminal amino acid sequence analysis of Psi1 did not reveal homology to any known sequences. By contrast, the N-terminal amino acid sequences of OprF of *P. aeruginosa* and *P. fluorescens* show high homology (Duchêne *et al.*, 1988; De Mot *et al.*, 1994) and the N-terminal amino acid sequences of many enterobacterial outer membrane

proteins can be aligned with homology (Jeanteur *et al.*, 1991).

The electrophoretic mobility and solubilization characteristics in SDS-PAGE differ between OprP and Psi1. The relative molecular mass of Psi1 in SDS-PAGE is 55 000 after solubilization in SDS at 100 °C. The mobility of Psi1 is not affected by solubilization temperature in four out of five *Pseudomonas* strains expressing this protein. In *P. fluorescens* strain Ag1, however, the protein migrates in part as a smear of M_r approximately 70 000–110 000 if samples are solubilized at room temperature. This smear may be attributed to the association of the protein with other cell envelope components, such as exopolysaccharides, which this strain produces in copious amounts (Boye *et al.*, 1995). OprP on the other hand is a trimer and is resistant to denaturation to monomers by SDS. It consequently appears as trimers of M_r approximately 97 000 in SDS-PAGE when not heated in SDS prior to electrophoresis, whereas the monomer, obtained by solubilization at 88 °C, migrates at M_r 48 000 (Poole *et al.*, 1987). In conclusion, the above data on amino acid composition, amino acid sequence, electrophoretic mobility and solubilization properties suggest that Psi1 is not closely related to OprP. However, we cannot at this point exclude the possibility that OprP of *P. aeruginosa* and *P. fluorescens* display considerable N-terminal heterogeneity, for example by the addition of an extra N-terminal domain to the *P. fluorescens* protein. This could explain the observed differences in amino acid sequence and electrophoretic mobility between OprP and Psi1.

The antiserum raised against Psi1 from strain Ag1 cross-reacts with only four other *P. fluorescens* strains among the 24 tested strains representing *Pseudomonas* rRNA homology group I and also does not react with phosphate-starvation-induced proteins from *E. coli*. Previously, Kragelund & Nybroe (1994) reported that the number and mobility of phosphate-starvation-induced outer-membrane proteins varied considerably among *P. fluorescens* and *P. putida* strains. One of these strains, *P. fluorescens* DF57, expressed a M_r 55 000 protein which, however, did not react with Psi1 antibody. Taking into account that OprP-like molecules have been characterized in some *P. fluorescens* strains (Poole & Hancock, 1986; Poole *et al.*, 1987; Siehnel *et al.*, 1990), the conclusion that can be drawn from the available data is that phosphate-starvation-induced outer-membrane proteins within *P. fluorescens* are heterogeneous.

Immunological detection of individual phosphate-starved cells

More information about the physiological responses of bacteria in natural habitats might be obtained by studying environmentally regulated gene expression under controlled laboratory conditions, and then developing systems which can allow us to detect expression of the selected genes under complex environmental conditions. Studies of bacterial physiology, such as during

starvation, in pure culture experiments have already revealed a considerable physiological heterogeneity between individual cells (Schaule *et al.*, 1993; Eberl *et al.*, 1996), making it crucial to establish reporter systems which can monitor the expression of specific functions at the single-cell level.

In the present study, we have developed an efficient and reliable cell-specific reporter system for phosphate limitation based on immunological recognition of Psi1. Initial attempts to visualize Psi1 by immunofluorescence microscopy of intact cells were unsuccessful (data not shown). This could indicate that the protein is shielded by, for example, bulky lipopolysaccharide (LPS) or exopolysaccharide. The O antigen in LPS has been reported to shield outer-membrane proteins (van der Ley *et al.*, 1986; Zaat *et al.*, 1994). Treatment with EDTA, which has previously been used to unmask outer-membrane proteins in pseudomonads (Zaat *et al.*, 1994; Kragelund *et al.*, 1996), did not improve the detection of Psi1, indicating that surface-exposed epitopes were not targeted by the antibody. However, immunofluorescence microscopy of fixed, permeabilized cells yielded the required signal specific to phosphate-starved cells.

Immunological reporter systems provide a sensitive means of cell-specific detection. Hence, Zaat *et al.* (1994) developed a marker for phosphate starvation based on a PhoE-ColA hybrid protein. However this marker could only be detected in a mutant of *P. putida* strain WCS358 lacking LPS O side chains. More recently, toluene degradation by *P. putida* KT2442 was visualized by an immunological reporter system targeting LamB, presenting a corona virus epitope, under the control of a catabolic promoter (Cebolla *et al.*, 1996). The present Psi1-targeted system is directed against an indigenous protein, thus circumventing the need for genetic modifications of the target cells. We had hoped that the Psi1 reporter could be used to study broader populations of pseudomonads, but the narrow specificity of the antibody precluded this approach. Therefore, we are currently planning experiments to investigate phosphate availability for selected strains introduced into the soil environment.

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