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

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**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
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$_2$HPO$_4$, 14 mM KH$_2$PO$_4$, 0.4 mM MgSO$_4$, 7.6 mM (NH$_4$)$_2$SO$_4$, 1.7 mM sodium citrate supplemented with 1 ml trace element solution [1]). The trace element solution contained 20 mg CoCl$_2$, 6H$_2$O, 10 mg ZnSO$_4$.7H$_2$O, 1 mg CuCl$_2$.2H$_2$O, 2 mg NiCl$_2$.6H$_2$O, 3 mg Na$_2$MoO$_4$.2H$_2$O, 10 mg FeSO$_4$.7H$_2$O, and 26 mg MnSO$_4$.H$_2$O dissolved in 11 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 agglomeration. 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$_4$)$_2$MoO$_4$.4H$_2$O, 1 vol. 2.5 mM H$_2$SO$_4$, and 2 vols acetone, was added to 200 µl sample (containing 2.5–200 nmol phosphate). A$_{560}$ 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% bromphenol 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 µm 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 protein concentration in the supernatant was determined colorimetrically. Half of the supernatant was removed and the remaining half was used to prepare outer membranes. 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 µm 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
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### 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biovar</th>
<th>Strength of reaction* of antibody with protein from cells which were:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Solubilized at 100 °C prior to electrophoresis</td>
</tr>
<tr>
<td><em>P. fluorescens</em> Ag1</td>
<td>II</td>
<td>+ + + (55)</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ON24†</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> MM5†</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> MM6†</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DF8†</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DF7†</td>
<td>II</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DF61†</td>
<td>III</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DF23†</td>
<td>IV</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DF18†</td>
<td>IV</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ON13†</td>
<td>V</td>
<td>+ + (55)</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ON25†</td>
<td>V</td>
<td>+ + (55)</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DF17†</td>
<td>IV or VI†</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ON5†</td>
<td>IV or VI†</td>
<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DSM 50090T</td>
<td>I</td>
<td>-</td>
</tr>
<tr>
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<td>II</td>
<td>+ + + (55)</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DSM 50124</td>
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<tr>
<td><em>P. fluorescens</em> DSM 50415</td>
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<td>-</td>
</tr>
<tr>
<td><em>P. fluorescens</em> DSM 50148</td>
<td>V</td>
<td>+ + + (55)</td>
</tr>
<tr>
<td><em>P. putida</em> DSM 291T</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td><em>P. putida</em> DSM 50222</td>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td><em>P. stutzeri</em> DSM 5190T</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> DSM 50071T</td>
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<td><em>P. chlororaphis</em> DSM 50083T</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. syringae</em> ATCC 19310T</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>E. coli</em> DSM 498</td>
<td>-</td>
<td>-</td>
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* + + +, Strong reaction; + +, medium reaction; +, weak reaction; -, no reaction. Figures in parentheses are $10^{-3} \times M$, 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 μm) 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 μl 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 antisera. Antiserum against purified protein
was made by immunizing two mice (strain Scs:CF1) sub-
cutaneously 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. Immuniz-
ation 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 NaCl (15 mM) was added as a preservative.

Immunoblotting. SDS-PAGE and electrophoretic transfer 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 antisera. The
antisera 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, 0.5% 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 immuno-
globulins (1:1000 in dilution buffer) (DAKO). After three
washes, colour was developed with 0.1 mg nitroblue tet-
razolium ml⁻¹ (Sigma), 0.06 mg 5-bromo-4-chloro-3-indolyl
phosphate ml⁻¹ (Sigma), 20 mM MgCl₂ in 100 mM ethanol-
amine, pH 9.6.

Dot-immunoblotting. Samples were collected on a nitro-
cellulose membrane by filtration. The membrane was immuno-
stained 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 tem-
perature before incubation with antibodies.

Immunofluorescence microscopy. The procedure for immuno-
fluorescence 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 im-
munoglobulin (DAKO) diluted 1:20.

RESULTS

Purification of Ps1

Outer-membrane preparations from exponentially growing and from phosphate-starved P. fluorescens Ag1
were analysed by SDS-PAGE. A protein of M, 55000, which we called phosphate-starvation-inducible protein
1 (Ps1), appeared under phosphate starvation (Fig. 1, compare lanes 1 and 2) but not under either carbon or
nitrogen starvation (data not shown). When reduced, Ps1 appeared to have a slightly higher M, approximately
56000, than non-reduced Ps1 (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 Ps1 protein was unaltered, but the
Ps1 protein band was less intense.

The M, 55000 form of Ps1 was purified from outer
membranes by elution from nitrocellulose membranes
after electrotransfer from SDS-polyacrylamide gels. The
protein preparation obtained was electrophotically 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 Ps1 protein migrated at M, 56000, whether heated or not
prior to electrophoresis (Fig. 2, lanes 3 and 4). The
M, 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 Ps1 was approxi-
mately 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, 55000 and the 56000 forms of Ps1 are
marked by arrows. The migration positions of M, standards are
indicated.
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 OprF of *P. aeruginosa* and PhoE of *Escherichia coli* and of the major outer-membrane protein OprF of *P. aeruginosa* 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: AVDAKLNLKANGQISASQYTELQAEALKDQKEKQIAQ. A homology search was carried out with all available sequence databases but did not reveal any homology to known proteins.

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

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>Psi1*</td>
</tr>
<tr>
<td>Asp/Asn</td>
<td>14-5</td>
</tr>
<tr>
<td>Thr</td>
<td>6-3</td>
</tr>
<tr>
<td>Ser</td>
<td>6-7</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>11-6</td>
</tr>
<tr>
<td>Pro</td>
<td>2-0</td>
</tr>
<tr>
<td>Gly</td>
<td>11-4</td>
</tr>
<tr>
<td>Ala</td>
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<tr>
<td>Cys</td>
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<td>Val</td>
<td>4-9</td>
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<tr>
<td>Met</td>
<td>0-9</td>
</tr>
<tr>
<td>Ile</td>
<td>3-9</td>
</tr>
<tr>
<td>Leu</td>
<td>8-2</td>
</tr>
<tr>
<td>Tyr</td>
<td>4-0</td>
</tr>
<tr>
<td>Phe</td>
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<td>Lys</td>
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</tr>
<tr>
<td>Arg</td>
<td>4-1</td>
</tr>
<tr>
<td>Trp</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.
† 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.
|| Derived from the nucleotide sequence of the protein (Overbeek et al., 1983).

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 preparations from *P. aeruginosa* Ag1. The antiserum recognized a protein of *M* ~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* ~approximately 70000–110000~, appeared in addition to the *M* ~55000~ band (Fig. 3, lane 3). When tested against a number of *P. aeruginosa* strains representing all biovars of this species and against a panel of type strains from RNA homology
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 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...
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and subsequently analysing the cells by immunoblotting to detect both Psil and the constitutive marker OprF. When cells were transferred to phosphate-free medium, Psil could be detected by immunoblotting after approximately 70 min. To ensure detection of Psil, 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 Psil was induced at phosphate concentrations between approximately 0.08 and 0.13 mM (Fig. 6).

DISCUSSION

Characterization of the phosphate-starvation-inducible protein Psil

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, Psil, from *P. fluorescens* strain Ag1. The Psil protein from strain Ag1 is induced during phosphate deprivation and is likely to be a member of the phosphate-starvation (Pho) regulon (Siehn et al., 1990; Siehn et al., 1992) but the protein is not a general starvation marker. Psil 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*, 55,000 Psil protein is exclusively localized in the outer membrane. In *P. aeruginosa*, the *M*, 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 Psil could represent a *P. fluorescens* homologue to OprP. However, the amino acid compositions of Psil and OprP were distinctly different and N-terminal amino acid sequence analysis of Psil did not reveal homology to any known sequences. By contrast, the N-terminal amino acid sequences of OprP 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 Psil. The relative molecular mass of Psil in SDS-PAGE is 55,000 after solubilization in SDS at 100 °C. The mobility of Psil 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*, 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*, 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*, 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 Psil 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 Psil.

The antiserum raised against Psil 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*, 55,000 protein which, however, did not react with Psil antibody. Taking into account that OprP-like molecules have been characterized in some *P. fluorescens* strains (Poole & Hancock, 1986; Poole et al., 1987; Siehn 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 Psil. Initial attempts to visualize Psil 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 Psil, 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 Psil-targeted system is directed against an indigenous protein, thus circumventing the need for genetic modifications of the target cells. We had hoped that the Psil 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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