Mol Gen Genet (1986) 202:403–409

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Isolation of a Tn*501* insertion mutant lacking porin protein P of *Pseudomonas aeruginosa*

Keith Poole and Robert E.W. Hancock

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

Summary. In order to demonstrate a role for anion-specific protein P channels in phosphate transport in *Pseudomonas aeruginosa* PAO, we wished to isolate a transposon insertion mutant deficient in protein P. A number of transposon delivery systems were tested which yielded, for the most part, whole plasmid inserts. Plasmid pMT1000 (Tsuda et al. 1984), a temperature-sensitive R68 plasmid carrying the transposon Tn*501*, was successfully employed in the isolation of a Tn*501* insertion mutant lacking protein P under normally inducing conditions. To identify the mutant deficient in protein P, a protein P-specific polyclonal antiserum was used. This mutant, strain H576, was deficient in high-affinity phosphate transport exhibiting a Km for uptake (3.60 ± 0.64 μM) almost ten times greater than that of the wild type strain (Km = 0.39 μM). There was, however, no change in the V_{max} for high-affinity phosphate transport as a result of the loss of protein P in this mutant. The protein P-deficiency of the mutant correlated with a growth defect in a phosphate-limited medium, resulting in an 18%–35% decrease in growth when compared with the wild type.

Key words: Tn*501* insertion – Porin protein P – *P. aeruginosa*

Introduction

The outer membranes of gram-negative bacteria function as permeability barriers, allowing the passage of hydrophilic molecules below a defined molecular weight (the exclusion limit) (Nikaido 1980). This permeation is a passive diffusion process mediated by specific membrane proteins termed porins, which generally form large water-filled channels through the outer membrane (Nikaido 1980; Hancock et al. 1979). Owing to their large size (1–2 nm in diameter), porins tend to be non-specific, exhibiting only weak ion selectively in reconstituted systems (Benz et al. 1985; Benz 1984). A noted exception is porin protein P of *P. aeruginosa* which forms small (0.6 nm) anion-selective channels in lipid bilayer membranes (Hancock et al. 1982; Benz et al. 1983).

The anion-specificity of protein P channnels has been attributed to the presence of positively charged lysine residues in the channel (Hancock et al. 1983), which form a binding site with, for example, a Kd for Cl^- of 30 mM (Hancock et al. 1983; R. Benz, personal communication). Based on physiological (Hancock et al. 1982; Poole and Hancock 1983) and mutant (Hancock et al. 1982; Poole and Hancock 1984), studies demonstrating that protein P was co-regulated with components of a high-affinity phosphate transport system in *Pseudomonas aeruginosa*, it was assumed that protein P played a role in this transport system. The recent demonstration (R.E.W. Hancock, unpublished result) of a high-affinity phosphate-binding site within the channel is consistent with this possiblity. In addition, the ability of phosphate to permeate protein P channels has been demonstrated in reconstituted lipid bilayer membranes (Hancock et al. 1982).

In order to confirm a role for protein P in in vivo phosphate transport it was necessary to isolate a mutant deficient in protein P. Because protein P has no readily identifiable phenotype associated with it, it was decided to generate such mutants using transposon insertion into the protein P gene. During the course of our search for a general purpose transposon insertion mutagenesis vehicle useful in *P. aeruginosa*, a temperature-sensitive plasmid vector, called pMT1,000 (R68::Tn*501*), was successfully employed in the isolation of Tn*501* insertion mutants in *P. aeruginosa* PAO (Tsuda et al. 1984). We describe here the use of this vector in the generation of a protein P-deficient mutant.

Materials and Methods

Media. Phosphate-sufficient and phosphate-deficient minimal sodium Hepes-buffered media were described previously (Hancock et al. 1982). L-broth used throughout this study consisted of 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 0.05% (wt/vol) NaCl. L-broth agar (L-agar) consisted of L-broth containing 2% (wt/vol) Bactoagar. Arginine (as L-arginine HCl) was used at 1 mM as required. Strains were routinely maintained on L-agar plates. Antibiotics were used in selective media at the following concentrations: tetracycline (Tc), 200 μg/ml; kanamycin (Km), 300 μg/ml; carbenicillin (Cb), 1,000 μg/ml; and mercuric chloride ($HgCl_2$), 15 μg/ml.

Bacterial strains and conjugation. Strains of *P. aeruginosa* and plasmids used in this study are listed in Table 1. Plasmid pMT1000 was introduced into strain H103 by conjugation with PAO1594 (pMT1000) on L-agar plates. Briefly, equal volumes of mid-log phase donor and recipient cells

Offprint requests to: R.E.W. Hancock

Table 1. Bacterial strains and plasmids

Strain	Description[a]	Source or reference
P. aeruginosa		
PAO1594	*met-28 ilv-202 rmo-53 str-1*	M. Tsuda
PAO1594 (pMT1000)	contains plasmid pMT1000	M. Tsuda
H103	PAO1 (wild type)	Hancock and Carey (1979)
H103 (pMT1000)	contains plasmid pMT1000	This study
H553	Tn*501* insertion mutant of H103 non-derepressible for alkaline phosphatase, phospholipase C, phosphate-binding protein and protein P	This study
H556	Tn*501* insertion mutant of H103 requiring arginine	This study
H576	Tn*501* insertion mutant of H103 non-derepressible for protein P	This study

Plasmids	Description	Source or reference
pAS8Rep-1	RP4-ColEl hybrid/rep (RP4)::Tn*7* (Tra^+ Cb^r Km^r Tc^s Tp^r)	Sato et al. (1981)
pUW942	pAS8Rep-1::Tn*501* (Tra^+ Cb^r Km^r Tc^s Tp^r Hg^r)	Weiss and Falkow (1983)
pKP100	putative pAS8Rep-1::Tn*5-132*[b] (Tra^+ Cb^r Km^r Tc^r Tp^r)	This study
pRK2013	RK2-ColEl hybrid Tra^+ Km^r	Figurski and Helinski (1979)
pUW964	pRK2013 (Kan::Tn*7*)::Tn*5* (Tra^+ Cb^s Km^r Tc^s Tp^r)	Weiss et al. (1983)
pME319	RPI^{ts} [Rep A^{ts} Rep B^{ts}] (carries Tn*1*) (Tra^+ Cb^r Km^r Tc^r)	Haas et al. (1981)
pME305	RPl with a 12 kb deletion in Km^r, primase and IS21 (Tra^+ Cb^r Km^s Tc^r)	Rella et al. (1985)
pME9	pME305::Tn*5-751*[d] (Tra^+ Cb^r Km^r Tc^r Tp^r)	Rella et al. (1985)

[a] Abbreviations: Cb^r, carbenicillin resistant; Km^r, kanamycin resistant; Tp^r, trimethoprim resistant; Tc^r, tetracycline resistant; Hg^r, mercuric chloride resistant; ts, temperature sensitive

[b] A derivative of Tn*5* carrying a Tc^r determinant in place of the Km^r determinant (Berg and Berg 1983)

[c] The temperature sensitive phenotype is due to a mutation in *trfA* (Rella et al. 1985)

[d] A derivative of Tn*5* carrying the Tp^r determinant of plasmid R751 (Rella et al. 1985)

(grown in L-broth) were mixed and pelleted by centrifugation. The supernatant was decanted and the pellet resuspended gently in 0.05 ml L-broth. The cells were spread over approximately one-third of the surface of a L-agar plate and incubated for 2 h at 30° C. The mating mixture was then resuspended in 1 ml phosphate sufficient medium, centrifuged and washed several times in the same medium. Transconjugants were selected at 30° C on phosphate-sufficient minimal medium containing 100 μg/ml Tc.

Tn501 insertion mutagenesis. *P. aeruginosa* H103 (pMT1000) was cultured overnight in L-broth at 30° C in the presence of $HgCl_2$. Dilutions were plated onto L-agar plates containing $HgCl_2$ and incubated at 42° C. Colonies growing up after 24 hours (insertion mutants) were picked onto grids on fresh low salt L-agar plates containing $HgCl_2$ and incubated once again at 42° C. After 24 h, these plates were then replica plated onto L-agar plates containing $HgCl_2$ and onto phosphate-deficient minimal plates, followed by incubation at 42° C. The replicas on rich medium were retained as a master set from which desired mutants, once identified, could be rescued. The replicas grown on the phosphate-deficient minimal plates were screened for protein P-deficient mutants.

Immunological techniques. The antigenic specificity and titre of the various antisera were determined using the enzyme-linked immunoadsorbant assay (ELISA) as described previously (Mutharia and Hancock 1983), except that antigen (LPS or protein) was used at 20 μg/ml final concentration. The Western immunoblot procedure has been described previously (Mutharia and Hancock 1983). In cases where a peroxidase-conjugate was used as the second antibody, azide was omitted from all buffers, and the blots were developed using the peroxidase substrate described below.

Preparation of a protein F-Sepharose affinity collumn. Protein F was partially purified according to the procedure of Yoshimura et al. (1983), omitting the column chromatography step. The resultant preparation, which had only minor contamination with protein H2, was approximately 90% pure as judged by SDS-polyacrylamide gel electrophoresis. The partially purified protein was passed across a Biogel P-10 (Biorad, Richmond, CA, USA) column (15 × 1.5 cm) equilibrated with 0.1 M $NaHCO_3$ (pH 8.3)/0.5 M NaCl/0.1% SDS. Peak fractions (measured at an absorbance of 280 nm) were pooled and the protein (approximately 3.5 mg) was cross-linked to CNBr-activated Sepharose 4B beads (approximately 0.5 g dry weight) as recommended by the manufacturer (Pharmacia, Upsalla, Sweden). The column was stored at 4° C in phosphate-buffered saline pH 7.4 (PBS) (Mutharia and Hancock 1983) containing 0.1% Triton X-100. The final column volume was approximately 1 ml. Prior to use, the column was washed exhaustively with PBS to remove excess detergent.

Preparation of a protein P-specific antiserum. Antibodies to protein P were raised in New Zealand White rabbits using the following imunization schedule. Protein P (50 μg), purified as described previously (Hancock et al. 1982), was injected subcutaneously at weekly intervals over a three week period. Following this, the rabbits were rested, without injection, for three weeks. This cycle of three weeks of weekly immunization followed by three weeks without injection was repeated twice more, before a final subcutaneous injection of protein P (50 μg) was given. For the first two injections, protein P (diluted in PBS) was mixed 1:1 with Freund's Incomplete Adjuvant (Difco, Detroit, MI, USA), otherwise it was injected in PBS alone. Two weeks after the final injection blood was collected and the serum obtained after centrifugation of clotted blood.

The resultant antiserum contained antibodies to lipopolysaccharide (LPS) and porin protein F as well as to protein P. Thus, in order to generate a protein P-specific antiserum it was necessary to remove these contaminating activities. The antiserum was first absorbed against whole cells of *P. aeruginosa* PAO1 strain H103 as follows. Cells from a 10 ml overnight culture in L-broth were harvested by centrifugation in a table top centrifuge and washed twice with Hank's Balanced Salt Solution (Gibco, Burlingon, Ont., Canada). The cell pellet was resuspended directly into 1.0 ml of the antiserum, placed in a 1.5 ml polypropylene centrifuge tube (Evergreen Scientific, Los Angeles, CA, USA) and incubated for 45 min at room temperature in an end-over-end shaker. The cells were then pelleted and the antiserum-containing supernatant adsorbed a second time against a fresh batch of washed cells. Whole cell adsorption effectively removed all antibody directed against smooth LPS as measured by ELISA and confirmed in Western immunoblots. There was, however, no decrease in antibody titre to protein F (or protein P).

The adsorbed antiserum (600 µl) was then incubated for 45 min at room temperature on a protein F-Sepharose column (2.5 × 0.7 cm). At the completion of the incubation period. the column was washed with 4 ml of PBS and the unbound antibodies collected in 400 µl fractions. Fractions containing antibodies to protein P, as determined by ELISA, were pooled to yield a protein F-adsorbed antiserum.

Adsorption of the antiserum on the protein F-Sepharose column facilitated removal of 99% of the antibody activity to protein F, with no decrease in antibody titre to protein P. The protein F-adsorbed antiserum, however, could only poorly distinguish between phosphate-limited cells producing protein P (eg. strain H103) and phosphate-limited cells defective in protein P production (eg. strain H553). Therefore, the protein F-adsorbed antiserum was subsequently adsorbed twice against phosphate-limited *P. aeruginosa* strain H533 cells as described earlier for strain H103. The resultant antiserum was protein P-specific (see Results).

Isolation of a protein P-deficient mutant. Bacterial clones resistant to $HgCl_2$ at 42° C and replica plated onto phosphate-deficient minimal plates, were transferred by contact onto nitrocellulose filter discs (Schleicher and Schuell Inc., Keene, NH, USA, type BA85, 82 mm). The nitrocellulose replicas were subsequently screened, by a modification of the procedure of Helfman et al. (1983), for the absence of protein P using the above-described protein P-specific antiserum.

Blotted filters were placed in individual Petri dishes in 10 ml of 50 mM Tris HCl (pH 7.4), 150 mM NaCl/5 mM $MgCl_2$ containing 3% bovine serum albumin (BSA) and shaken gently for 1 hour at 37° C. The filters were then washed twice at room temperature for 10 min in 10 ml of Tris-buffered saline [0.9% NaCl/10 mM Tris-HCl (pH 7.4); Towbin et al. 1979] with shaking, followed by three 5 min washes in 10 ml of PBS. Protein P-specific antiserum (with an ELISA titre of 2,000, indicating that antibodies to protein P were detected at a 1/2,000 dilution of the antiserum) was diluted 1:249 in PBS containing 3% BSA (10 ml) and then incubated on the filters overnight at room temperature with shaking. The filters were subsequently washed three times for 10 min at room temperature in 10 ml PBS. Affinity purified goat anti-rabbit IgG(H + L)-peroxidase conjugated antibody (Cappel Laboratories, West Chester, PA, USA) diluted 1:999 in 10 ml PBS containing 3% BSA, was then incubated on the filters at 37° C, again with shaking. After 2 h of incubation, the filters were washed twice for 10 min each in 10 ml of PBS at room temperature, followed by three washes of 10 min each in 10 ml of Tris-buffered saline. Peroxidase substrate [30 mg 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO, USA) in 10 ml of methanol/50 ml of Tris-buffered saline/0.02 ml H_2O_2 (30% vol/vol)] was then added (10 ml/filter) and the filters incubated at 37° C until colour developed. Those colonies failing to develop colour were identified and picked from the master plates and screened for the absence of protein P in sodium dodecyl sulphate (SDS)-polyacrylamide gels of phosphate-limited cell envelopes.

Transport assays. The uptake of ^{32}P-orthophosphate (Amersham, Oakville, Ont., Canada) by whole cells was assayed as described previously (Poole and Hancock 1984), except that cells were prewarmed for 5 min at 37° C with shaking prior to the assay.

Characterization of whole cell and cell envelope protein. Whole cell protein extracts were obtained as described previously (Nicas and Hancock 1980). The preparation of cell envelopes has been described previously (Hancock and Carey 1979). TX-100 insoluble cell envelopes were prepared by extraction with 2% TX-100/20 mM Tris-HCl (pH 8.0) as described (Hancock and Carey 1979). Examiniaton of whole cell and cell envelope proteins was performed by SDS-plyacrylamide gel electrophoresis, using a 12% (wt/vol) acrylamide running gel (Hancock and Carey 1979).

Results

Preparation of a protein P trimer-specific antiserum.

Protein P forms SDS-stable trimers (Angus and Hancock 1983) which dissociate (to monomers) when heated at temperatures above 60° C (Hancock et al. 1982). A polyclonal rabbit antiserum raised against purified protein P trimers (see Materials and Methods) reacted specifically with the native trimer form of the protein (Fig. 1 A, lane 3), exhibiting no reaction with the heat dissociated monomers (Fig. 1 A, lane 4). The smearing pattern evident in the reaction of the antibody with electrophoresed protein P trimers suggested some heterogeneity. This may be due to an association of the trimer form of the protein with LPS or due to aggregation of the trimers. Nevertheless all of the material in the smear reacting with the antibody was protein P as confirm by the ability to convert this material to protein P monomers by heating.

The specificity of the polyclonal antiserum to protein P was demonstrated by the ability of the antiserum to react with a component present in envelopes from phosphate-starved cells (Fig. 1 A, lane 2) but which was absent in envelopes from phosphate-sufficient cells (Fig. 1 A, lane 1). The reaction profile was very similar to that seen with purified protein P trimers (Fig. 1 A, lane 3).

Tn501 mutagenesis of P. aeruginosa

In our search for a suitable vehicle for use in the transposon insertion mutagenesis of *P. aeruginosa* PAO1, a number of vectors were tested (see Table 1). One class, which in-

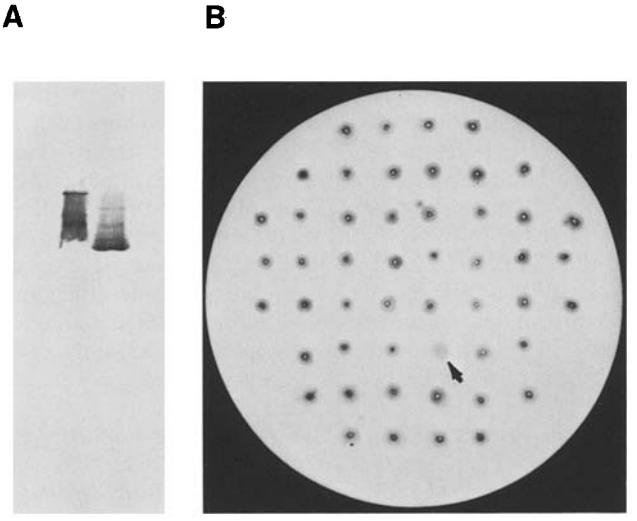

Fig. 1. A Western immunoblot of *P. aeruginosa* PAO1 strain H103 cell envelopes and purified protein P. Cell envelopes from phosphate-sufficient cells (lane 1), phosphate-limited cells (lane 2) and purified protein P (lanes 3 and 4) were separated on SDS-polyacrylamide gel electrophoretograms after solubilization at 23° C (lanes 1–3) or 88° C (lane 4) for 10 min. After electrophoretic transfer to nitrocellulose, the blots were interacted with the protein P-specific polyclonal antiserum and subsequently immunostained using a peroxidase-conjugated goat-anti-rabbit IgG antibody and a histochemical stain for peroxidase (see Materials and methods). **B** A colony immunoblot showing the interaction of the protein P-specific polyclonal antiserum with phosphate-limited Tn*501* insertion mutants of *P. aeruginosa* PAO1 strain H103. The protein P-deficient mutant, strain H576, is indicated by the arrow head

cluded plasmids pME9 and pME319, were temperature sensitive for maintenance due to mutations. Selection for transposon-encoded resistance at the non-permissive temperature (42° C) resulted in insertions into the PAO chromosome. Recovery of the transposable elements at the non-permissive temperature was usually (>98%) associated with the recovery of all plasmid antibiotic resistance markers as well, indicating that the entire plasmid had probably inserted. The observation that inserts generated by pME9 consistently involved all plasmid markers was in contrast to results obtained in another strain by Rella et al. (1985).

The second class of vectors tested included plasmids pUW942 (::Tn*501*), pUW964 (::Tn*5*), pASBRep-1 (::Tn*7*) and pKP100 (::Tn*5–132*) which were hybrids comprising the broad host range transfer functions of the IncP-1 plasmids (eg. RP1) and the narrow host range replication functions of the ColEl-like plasmids. Thus these vectors could be transferred from *E. coli* to *P. aeruginosa* but were unable to replicate in this recipient (Bagdasarian et al. 1979). Selection for transposon-encoded resistances in *P. aeruginosa* PAO1 strains mated with *E. coli* strains harbouring these plasmids revealed colonies with insertions in the chromosome. Almost without exception, these insertion events involved the whole plasmid, as indicated by the recovery of all plasmid-encoded resistance markers.

Plasmid pMT1000, recently described by Tsuda et al. (1984), is a temperature-sensitive R68 plasmid carrying a Tn*501* element. Insertion mutants in *P. aeruginosa* can be readily selected on mercuric chloride containing plates at 42° C. After raising the incubation temperature of *P. aeruginosa* PAO1 strain H103 (pMT1000) to the restrictive temperature (42° C), colonies resistant to $HgCl_2$ were isolated at a frequency of $>1\times10^{-3}$/viable cell. Of these approximately 30% apparently represented whole plasmid inserts, in that they were resistant to Cb, Tc and Km, as well as to $HgCl_2$, and this proportion decreased to <15% after a single passage on $HgCl_2$-containing plates. The remainder of the mercury resistant colonies were sensitive to Cb, Tc and Km. This, together with the high frequency of isolation suggested that they were Tn*501* insertion mutants.

Examination of colonies growing on $HgCl_2$-containing plates at 42° C revealed the existence of two colony morphologies which could be correlated to the type of insertion event which had occurred in these clones in the rescue of the Tn*501* element. Colonies containing whole plasmid inserts (Hg^r, Tc^r, Km^r, Cb^r at 42° C) were typically flat, translucent and irregularly shaped. Colonies containing a resolved Tn*501* insert (Hg^r, Tc^s, Km^s, Cb^s at 42° C) were opaque, dome-shaped and generally circular, typical of wild type.

The isolation, in the majority of cases, of resolved Tn*501* inserts in *P. aeruginosa* PAO1 strain H103 meant that the Tn*501* insertion mutagenesis was significantly simpler than published procedures (Tsuda et al. 1984) requiring no curing of plasmid sequences. In addition, we confirmed the mutagenic capability of plasmid pMT1000 (Tsuda et al. 1984) in *P. aeruginosa* PAO1 strain H103 by the isolation of auxotrophs (frequency $=2\times10^{-3}/Hg^r$ colony), mutants deficient in pigment production ($6\times10^{-4}/Hg^r$ colony), and a number of *pho* regulon mutants, including phosphate-binding protein-deficient mutants ($2\times10^{-4}/Hg^r$ colony), alkaline phosphatase constitutive mutants ($1\times10^{-3}/Hg^r$ colony) and alkaline phophatase deficient mutants ($3.3\times10^{-4}/Hg^r$).

Isolation of a Tn501-induced protein P-deficient mutant.

Of 3,200 mercury resistant colonies screened, only one failed to react strongly with the protein P-specific antiserum in the colony blot assay (Fig. 1 B). SDS-polyacrylamide gel electrophoresis of cell envelopes of this mutant (designated strain H576), grown under phosphate-deficient conditions, confirmed the absence of detectable protein P (Fig. 2, lane 5). In contrast, the parent strain H103 grown under the same conditions produced large quantities of protein P (Fig. 2, lane 3). Western immunoblots of electrophoretically-separated cell envelope and whole cell proteins confirmed the absence of detectable protein P in phosphate-limited mutant cells using both a protein P trimer-specific and monomer-specific antiserum (data not shown). The mutant, like its parent, was normally derepressible for alkaline phosphatase and phospholipase C under conditions of phosphate deficiency. In addition, the presence of the phosphate starvation-inducible periplasmic phosphate-binding protein in shock fluids and whole cell extracts of the mutant was confirmed using SDS-polyacrylamide gel electrophoresis and Western immunoblotting with a phosphate-binding protein-specific antiserum (data not shown). These results confirmed the specific loss of protein P in this mutant, and distinguished this strain from a class of mutants isolated previously which were pleiotropically deficient in the phosphate-regulated components of *P. aeruginosa*, including protein P (Hancock et al. 1982; Poole and Hancock 1984).

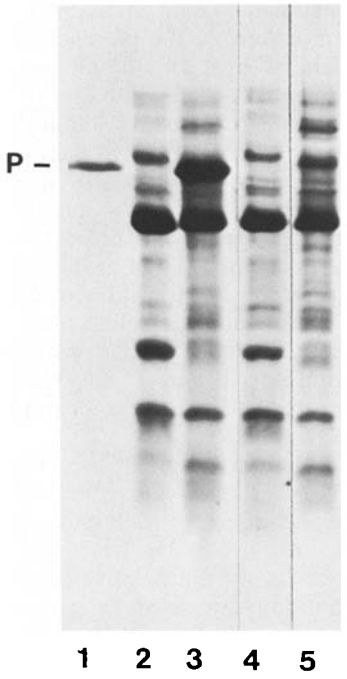

Fig. 2. SDS-polyacrylamide gel electophoretogram of purified protein P (lane 1) and of the following Triton-insoluble cell envelope preparations: lane 2, phosphate-sufficient H103 cells; lane 3, phosphate-deficient H103 cells; lane 4, phosphate-sufficient H576 cells; lane 5, phosphate-deficient H576 cells. All preparations were solubilized at 88° C prior to electrophoresis such that protein P ran as the monomer

Phosphate transport

Phosphate transport in *P. aeruginosa* is characterized by the presence of two major systems of uptake, a low-affinity system and a phosphate binding protein-dependent high-affinity system (Poole and Hancock 1984). When stationary phase, phosphate-starved cells of *P. aeruginosa* were pre-incubated at 37° C with aeration for only 5 min prior to transport assays, it was possible to examine high-affinity phosphate transport alone, since it was found necessary to incubate cells for longer periods (15–25 min) at 37° C for the low-affinity uptake system to become operative (data not shown). Thus it was possible to precisely examine what role, if any, protein P played in high-affinity phosphate transport by comparing phosphate uptakte in the protein P-deficient mutant with that of its parent strain H103.

Compared with the wild type parent strain, the protein P-deficient mutant was significantly defective in phosphate transport, exhibiting a K_M for high-affinity transport almost 10 times greater than that of the parent (Table 2). No effect on the V_{max} of the system was seen, however, as a result of the loss of protein P in the mutant (Table 2). This confirmed the involvement of protein P in high-affinity phosphate transport of *P. aeruginosa*.

Although it was not possible to accurately determine the kinetic parameters of low affinity phosphate transport in the mutant, owing to the presence of a high affinity uptake component (Poole and Hancock 1984), the rates of phosphate transport measured in the mutant (H576) and wild type (H103) were comparable at higher concentration of phosphate (>25 μM) suggesting that low affinity phosphate uptake was not affected by the protein P deficiency of the mutant.

Table 2. Kinetics of phosphate transport

Strain	Km (μM)	V_{max} (μmol/min/mg cell protein)
H103	0.39±0.07	5.34±0.59
H576	3.60±0.64	5.56±0.66

Initial rates of phosphate transport at various concentrations of phosphate were plotted as an Eadie-Hofstee plot, from which kinetic parameters were derived by least squares analysis. The results are the mean values±standard deviations of four experiments

Table 3. Growth of *P. aeruginosa* in phosphate-limited medium[a]

Experiment	Strain[b]	Doubling time[c]
1	H103	12.82±1.18
	H576	15.87±0.60
2	H103	9.98±0.90
	H576	15.43±2.57
3	H556	13.55±0.33
	H576	19.60±1.62
4	H556	11.27±0.53
	H576	13.67±0.33

[a] Overnight cultures, grown in phosphate-deficient medium, were resuspended in triplicate in Hepes-buffered minimal medium containing 50 μM phosphate at an absorbance at 600 nm (A_{600}) of 0.20 and growth measured by the increase in A_{600}

[b] H103, wild type PA01; H556, arginine requiring Tn*501* insertion mutant; H576, Tn*501* insertion mutant deficient in protein P

[c] Doubling times in hours represent the reciprocal of growth rate, μ, calculated from a plot of ln A_{600} vs. time (min) using least squares analysis. Results are expressed as the mean doubling times±standard deviation for three cultures (see above). Variations from experiment to experiment in growth rates obtained for a given strain reflect technical difficulties in obtaining precisely the same degree of phosphate limitation every time. Within a given experiment, however, the degree of limitation was the same for each strain

Growth in low phosphate medium

We wished to determine if the transport differences attributable to a lack of protein P were significant in terms of the growth capabilities of the cell under limiting phosphate conditions (under which conditions protein P is normally derepressed; Hancock et al. 1982). Thus strain H103 wild type and strain H576 mutant cells were grown in phosphate-deficient medium for 14–16 h to deplete internal phosphate pools and thus make growth dependent on transported phosphate. These cells were then placed in a Hepes-buffered minimal medium containing 50 μM phosphate. Typically, we observed a lag period of 30–45 min followed by logarithmic growth for 2–4 h at a very reduced rate after which the cell stopped growing. Determination of the rate of growth during this period showed that the protein P-deficient mutant grew more slowly than its wild type parent strain H103 (Table 3).

To eliminate possible growth differences attributable to the presence of a Tn*501* element in the chromosome of

the protein P deficient-mutant, an arginine auxotroph, strain H556, obtained by Tn*501* insertion mutagenesis, was used as the protein P-derepressible control. Again, the mutant lacking protein P exhibited a slower rate of growth than the strain producing wild type levels of protein P (Table 3). The 18–35% increase in doubling time of the protein P mutant strain stressed the importance of protein P channels in the outer membrane of *P. aeruginosa* cells growing in a phosphate-limited environment.

Discussion

Transposon insertion mutagenesis is well documented in *E. coli* and other genera (eg. *Salmonella* and *Rhizobium*) where several plasmid (Kretschner and Cohen 1977; Beringer et al. 1978; Rostas et al. 1984) and phage (Bukhari and Zipser 1972; Kleckner and Botstain 1977; Shaw and Berg 1979) vectors have been described. Unfortunately, fewer suitable delivery vehicles are available for use in the transposon mutagenesis of *P. aeruginosa*, and those described suffer from any deficiencies. The most common problems include failure to resolve transposon-mediated cointegrates, which must be cured to leave a single copy of the transposon in the mutated gene (Haas et al. 1981; Nicas and Iglewski 1984; Tsuda et al. 1985; this study), low frequency recovery of transposon inserts in the chromosome (Haas et al. 1981; Rella et al. 1985), insertion-site specificity (Krishnapillai et al. 1981; Caruso and Shapiro 1982), and the intrinsic high-level drug resistance of the host and high frequency of spontaneous drug resistant mutants (Mitsuyoshi et al. 1983; Bryan et al. 1980; Day et al. 1984) both of which can mask transposon-encoded drug resistance.

The transposon delivery vehicle plasmid pMT1000 (Tsuda et al. 1984), which employs Tn*501* as the mutagenic agent, has several advantages over other transposon mutagenesis systems used in *Pseudomonas aeruginosa*. It generates a substantial number of resolved Tn*501*-mediated insertion events in *P. aeruginosa* strain H103 (10^{-3} per bacterium), leaving Tn*501* in the chromosome in the absence of plasmid sequences (Tsuda et al. 1984; this study). Although whole plasmid insertion events mediated by Tn*501* can be cured, in most instances to leave a single copy of the transposon in the chromosome (Tsuda et al. 1984) this represents further manipulation and in a minority of cases the plasmid sequences can not be cured (Tsuda et al. 1984). Although some preferred sites for Tn*501* insertion do apparently exist (Tsuda et al. 1984), the generation of several distinct auxotrophic mutants (Tsuda et al. 1984) and a number of *pho* regulon mutants (this study) using Tn*501* mutagenesis suggests that this transposon can insert into many genes, making it useful as a general mutagenesis tool, in contrast to the transposons Tn*1* and Tn*7* which have been demonstrated to insert at fairly specific sites on the *P. aeruginosa* chromosome (Krishnapillai et al. 1981; Caruso and Shapiro 1982). The use of Tn*501* also avoided problems associated with the selection of spontaneous resistant mutants and the high intrinsic resistance of *P. aeruginosa* to certain antibiotics, both of which can mask transposon-encoded resistance. Tn*501* inserts are easily selected on a little as 10 μg/ml of $HgCl_2$ and the frequency of spontaneous mercury resistant mutants observed is less than 10^{-9} (K. Poole, unpublished results).

The ability of Tn*501* to transpose to the *P. aeruginosa* chromosome has been demonstrated (Tsuda et al. 1984). Although we have not confirmed directly the presence of a Tn*501* element in the protein P gene of our mutant, we can find no other explanation to account for the observed properties of the mutant. It was isolated by examination of only 3,200 Hg^{2+} resistant cells, well above the expected frequency due to spontaneous mutation. The loss of all plasmid antibiotic resistance markers in the mutant supported the contention that only Tn*501* was present in the chromosome in this strain. Furthermore, the specificity of the mutation, as demonstrated by the retention of all *pho*-regulon constituents tested, (including alkaline phosphatase, phospholipase C, the phosphate-binding protein and both major phosphate transport systems), suggested that the transposed Tn*501* is probably inserted into or near the structural gene for protein P.

An important feature of the selection system used for the protein P-deficient mutant was the circumvention of the lack of an easily selectable phenotype for such mutants by using a specific antiserum. Previous studies which employed an immunological probe involved a positive selection procedure, whereby an antibody was used, for example, to identify a cloned gene product (Helfman et al. 1983). In combination with the use of a suicide vector which allows the exclusive selection, under approprate conditions, of transposon-generated mutants, the negative selection procedure described here could be useful in the isolation of mutants lacking any cellular component for which a monospecific or monoclonal antibody can be prepared.

Our reason for wishing to isolate a mutant in protein P was to confirm our previous hypothesis concerning its role in phosphate transport. The isolation here of a mutant deficient in protein P and altered in the kinetics of high-affinity phosphate transport, confirmed this role. Furthermore, the correlation of a phosphate transport deficiency with a growth defect under limiting phosphate conditions underlined the importance of this channel to *P. aeruginosa* cells growing in a nutritionally dilute environment. Although the growth rates found in studies comparing wild type and mutant strains were small (doubling times of 10–20 h) compared with growth rates obtainable in rich or phosphate-sufficient media (doubling times of 45–60 minutes) (Hancock et al. 1982), they probably more acurately reflect the growth of *P. aeruginosa* in its natural environment, the soil, where nutrient limitation might be common. Interestingly, Korteland et al. (1982) have demonstrated that a mutant deficient in the phosphate-regulated PhoE porin protein of *E. coli* exhibitis a 10-fold higher Km for phosphate uptake. Unfortunately, this result was obtained in a porin-deficient background, rather than a background wild type for the major porin proteins as was the case in this study. As such, it is not possible to conclude whether the increase in Km for phosphate in the PhoE-deficient strain reflects a specific role for PhoE in phosphate transport, or whether it simply reflects the overall porin deficiency of the PhoE mutant strain. It would be interesting to see if a PhoE deficiency had any effect on phosphate uptake in a strain also expressing the major porins OmpF and OmpC.

Acknowledgements. We thank M. Tsuda for his generous gift of plasmid pMT1000. This work was supported by a Natural Science and Engineering Research Council of Canada (NSERC) operating grant to REWH. KP is supported by an NSERC postgraduate scholarship.

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Communicated by J.W. Lengeler

Received September 23 / December 2, 1985