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Phosphate-Starvation-Induced Outer Membrane Proteins of Members of the Families *Enterobacteriaceae* and *Pseudomonodaceae*: Demonstration of Immunological Cross-Reactivity with an Antiserum Specific for Porin Protein P of *Pseudomonas aeruginosa*

KEITH POOLE AND ROBERT E. W. HANCOCK*

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

Received 23 October 1985/Accepted 2 December 1985

Bacteria from members of the families *Enterobacteriaceae* and *Pseudomonadaceae* were grown under phosphate-deficient (0.1 to 0.2 mM P_i) conditions and examined for the production of novel membrane proteins. Of the 17 strains examined, 12 expressed a phosphate-starvation-induced outer membrane protein which was heat modifiable in that after solubilization in sodium dodecyl sulfate at low temperature the protein ran on gels as a diffuse band of higher apparent molecular weight, presumably an oligomer form, which shifted to an apparent monomer form after solubilization at high temperature. These proteins fell into two classes based on their monomer molecular weights and the detergent conditions required to release the proteins from the peptidoglycan. The first class, expressed by species of the *Pseudomonas fluorescens* branch of the family *Pseudomonadaceae*, was similar to the phosphate-starvation-inducible, channel-forming protein P of *Pseudomonas aeruginosa*. The second class resembled the major enterobacterial porin proteins and the phosphate-regulated PhoE protein of *Escherichia coli*. Using a protein P-trimer-specific polyclonal antiserum, we were able to demonstrate cross-reactivity of the oligomeric forms of both classes of these proteins on Western blots. However, this antiserum did not react with the monomeric forms of any of these proteins, including protein P monomers. With a protein P-monomer-specific antiserum, no reactivity was seen with any of the phosphate-starvation-inducible membrane proteins (in either oligomeric or monomeric form), with the exception of protein P monomers. These results suggest the presence of conserved antigenic determinants only in the native, functional proteins.

The cell envelope of gram-negative bacteria consists of a cytoplasmic membrane, a peptidoglycan layer, and an outer membrane. The outer membrane, which comprises phospholipids, lipopolysaccharide, and protein (16), functions as a permeability barrier, allowing the passage of hydrophilic molecules below a defined molecular weight (the exclusion limit) (18). This permeation is mediated by a class of proteins, termed porins, which form water-filled diffusion channels across the outer membrane (9, 18). Porins generally exist as native functional trimers (1, 18) which are resistant to denaturation by sodium dodecyl sulfate (SDS) (11, 16) but can be dissociated (to nonfunctional monomers) upon heating at temperatures greater than 60°C (11, 16). Heat-dissociated porin monomers derived from numerous members of the family *Enterobacteriaceae*, including the OmpF, OmpC, and PhoE porin monomers of *Escherichia coli*, have been demonstrated to cross-react immunologically (13, 21). This implies that porin structure is, to some degree, conserved during evolution. However, the observation that porin trimers and porin monomers express different antigenic determinants (14) makes the relevance of these studies to native porins questionable. Nonetheless, the possibility that proteins displaying a similar function might be structurally related is intriguing.

Protein P, an outer membrane protein of *Pseudomonas aeruginosa*, is derepressed in wild-type cells in response to phosphate limitation (11). A trimer in its native state (1), it can be dissociated to monomers with molecular weights of 48,000 upon heating at temperatures greater than 60°C (16). Reconstitution of the purified trimer protein into black lipid bilayer membranes results in the formation of small (0.6-nm diameter) water-filled channels specific for anions (11). Based on studies of the aceylated channel and the pH dependence of single-channel conductance, the anion specificity has been attributed to the presence of positively charged lysine residues in or near the mouth of the channel (12). Physiological (11, 22) and mutant (11, 23) studies demonstrated that protein P was coregulated with components of high-affinity phosphate transport, suggesting a role for protein P in phosphate transport in *P. aeruginosa*. The recent demonstrations that protein P possesses a phosphate-binding site (R. E. W. Hancock and R. Benz, manuscript submitted for publication) and that a protein P-deficient mutant is deficient in high-affinity phosphate uptake (K. Poole and R. E. W. Hancock, Mol. Gen. Genet., in press) has confirmed this role.

Phosphate-regulated outer membrane proteins which have been identified in other bacteria include the PhoE proteins of *E. coli* (19), *Salmonella typhimurium* (2), and *Enterobacter cloacae* (28) and a 36,000-molecular-weight (36K) protein of *Klebsiella aerogenes* (25). All except the 36K protein, which has not yet been examined, have been demonstrated to form weakly anion-selective channels (2, 3, 20, 28) consistent with their proven (15) or presumed roles in phosphate acquisition. We report here that a number of bacterial species demonstrate phosphate-starvation-induced outer membrane proteins which cross-react immunologically.

* Corresponding author.

TABLE 1. Bacterial strains

Organism	Description[a,b]	Growth temp (°C)	Reference or source
Pseudomonadaceae			
P. aeruginosa	PAO1 strain H103	37 or 37	8
P. aeruginosa	ATCC 19305^T	30 or 37	
P. putida	ATCC 12633^T	30	
P. fluorescens	ATCC 949	30	
P. chlororaphis	ATCC 9446^T	30	
P. aureofaciens	ATCC 13985^T	30	
P. cepacia	ATCC 25609^T	30	
P. pseudomallei	ATCC 24433^T	37	
P. acidovorans	Strain 29	30	29
P. stutzeri	ATCC 17588^T	30	
P. syringae	ATCC 19310^T	30	
P. solanacearum	ATCC 11696^T	30	
X. maltophilia	ATCC 13637^T (formerly *P. maltophilia*)	30 or 37	
Enterobacteriaceae			
E. coli K-12	Strain HMS174 OmpF$^+$ OmpC$^+$	30 or 37	R. A. J. Warren[c]
E. coli K-12	Strain JF700 OmpF$^-$ OmpC$^+$	30 or 37	6
E. coli K-12	Strain JF694 OmpF$^-$ OmpC$^-$ PhoE^c	30 or 37	7
S. typhimurium	Strain SL1906 OmpC$^+$ OmpD$^-$ OmpF$^+$	30 or 37	26
K. pneumoniae	ATCC 13883^T	30 or 37	
Enterobacter aerogenes	ATCC 13048^T	30 or 37	
Serratia marcescens	ATCC 13880^T	30 or 37	

[a] ATCC, American Type Culture Collection; only the relevant phenotypes are indicated. PhoE^c means constitutive for the production of PhoE due to the *nmpA* mutation (27).
[b] The superscript T indicates type strain.
[c] R. A. J. Warren, Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are described in Table 1. Phosphate-sufficient (1 mM potassium phosphate) and phosphate-deficient (0.2 mM phosphate) sodium-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered minimal media were prepared as described previously (11) with the following exceptions. When the culture organism was *Pseudomonas cepacia*, *Pseudomonas pseudomallei*, or *Pseudomonas acidovorans*, the phosphate-deficient medium contained 0.1 mM phosphate. When the culture organism was *Klebsiella pneumoniae*, *Enterobacter aerogenes*, or *Serratia marcescens*, the phosphate-deficient medium contained 0.15 mM phosphate. *Pseudomonas maltophilia* cultures were supplemented with 1 mM methionine. All media contained glucose (0.4% [wt/vol]) in addition to 20 mM succinate as the carbon sources. The growth temperatures of the various organisms are indicated in Table 1. Strains were routinely maintained on both L-agar plates (1% [wt/vol] tryptone–0.5% [wt/vol] yeast extract–0.05% [wt/vol] NaCl–2% [wt/vol] Bacto-Agar) and phosphate-sufficient minimal medium plates (phosphate-sufficient medium plus 2% [wt/vol] Bacto-Agar containing 0.4% [wt/vol] glucose and 20 mM succinate, and in the case of *P. maltophilia*, 1 mM methionine). Stationary-phase cultures (20 ml), obtained by inoculation of minimal media (phosphate sufficient or deficient) with organisms maintained on phosphate-sufficient minimal medium plates followed by growth overnight, were suspended in fresh minimal media (200 ml) and again grown overnight to yield the phosphate-sufficient and phosphate-deficient cells used in this study.

Cell fractionation and gel electrophoresis. The preparation of cell envelopes has been described previously (10). Triton X-100-insoluble cell envelopes were prepared by extracting cell envelopes once with 2% (wt/vol) Triton X-100–20 mM Tris-hydrochloride (pH 8.0) as described previously (10). Triton X-100-EDTA solubilization of cell envelopes has been described previously (11). Peptidoglycan-associated proteins were extracted from cell envelopes in 2% (wt/vol) SDS–0.5 M NaCl (30) or 2% (wt/vol) Triton X-100 after lysozyme digestion as described previously (10). SDS-polyacrylamide gel electrophoresis was performed as described previously (8) with a 12% (wt/vol) acrylamide running gel.

Two-dimensional (unheated by heated) gel electrophoresis was based on a method described previously (24). Samples solubilized at room temperature were electrophoresed on an SDS-polyacrylamide slab gel, and the lanes were excised (first dimension). The gel strips were then placed in screw-cap tubes containing 2% (wt/vol) SDS–20 mM Tris-hydrochloride (pH 6.8) and heated at 88°C for 10 min. The heated gel strips were laid horizontally across the top of a second SDS-polyacrylamide slab gel, sealed in place with 0.8% agarose (Bio-Rad Laboratories, Richmond, Calif.), and electrophoresed again (second dimension). Where indicated, urea was included at a final concentration of 6 M.

Immunological methods. The antigen specificity and the titer of the various antisera were determined by enzyme-linked immunosorbent assay as described previously (17) with a final concentration of 20 μg of antigen per ml in the wells. The Western immunoblot procedure, involving the electrophoretic transfer of SDS-polyacrylamide gel electrophoretograms to nitrocellulose and subsequent immunostaining, has been described previously (17).

Preparation of antisera. Antibodies specific for protein P trimers were prepared as described previously (Poole and Hancock, in press). Antibodies to protein P monomers were raised as follows. Purified protein P (11) was heated at 88°C for 15 min to promote the heat denaturation of trimers to form monomers (11). After cooling, the protein (20 μg), suspended in 0.1 ml of phosphate-buffered saline (pH 7.4) (17), was injected interperitoneally into BALB/c mice (Department of Microbiology breeding colony, University of

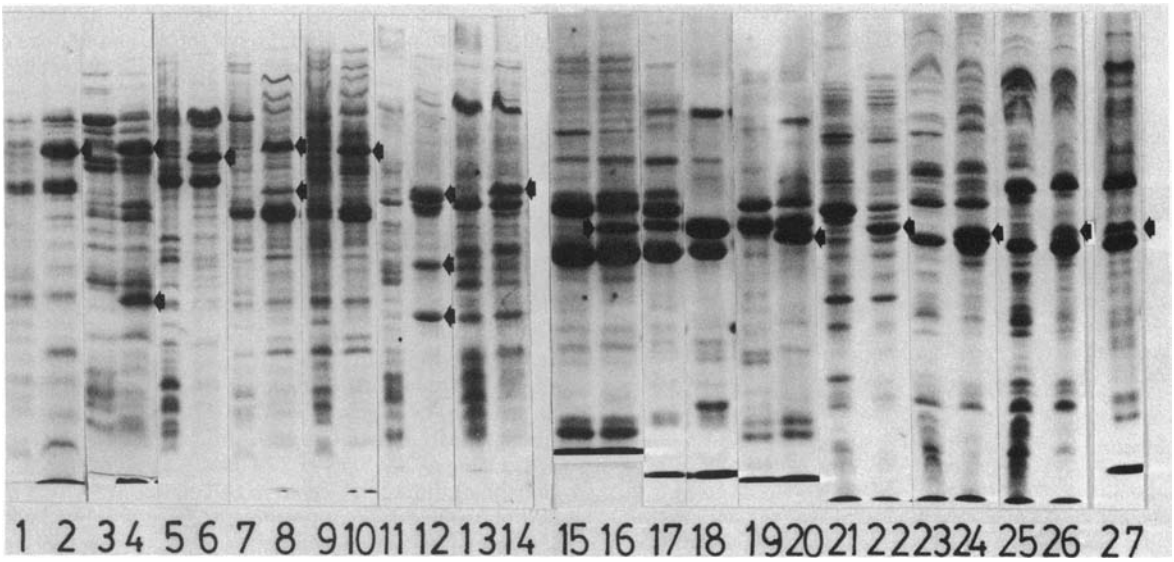

FIG. 1. SDS-polyacrylamide gel electrophoretogram of cell envelopes (lanes 1 to 14) and Triton X-100-insoluble cell envelopes (lanes 15 to 27). Unless indicated otherwise, odd-numbered lanes contain organisms grown in phosphate-sufficient medium and even-numbered lanes contain organisms grown in phosphate-deficient medium. Cells were prepared from lanes: 1 and 2, *P. aeruginosa*; 3 and 4, *P. fluorescens*; 5 and 6, *P. putida*; 7 and 8, *P. chlororaphis*; 9 and 10, *P. aureofaciens*; 11 and 12, *P. cepacia*; 13 and 14, *P. pseudomallei*; 15 and 16, *E. coli* K-12 strain JF700; 17, L-broth-grown *E. coli* K-12 strain HMS174; 18, L-broth-grown *E. coli* K-12 strain JF694; 19 and 20, *S. typhimurium* LT2; 21 and 22, *Serratia marcesens*; 23 and 24, *K. pneumoniae*; 25, *Enterobacter aerogenes*; 26 and 27, phosphate-deficient *Enterobacter aerogenes*. The arrows indicate the phosphate-regulated proteins. All samples were solubilized at 88°C prior to electrophoresis. The gel in lanes 15 to 27 contained urea at a final concentration of 6 M.

British Columbia, Vancouver, Canada). The injection was repeated on days 14, 28, 35, 42, 45, and 50. The blood was collected 7 days after the final injection, and the serum was obtained after centrifugation of clotted blood. Antibodies to lipopolysaccharide, as measured by an enzyme-linked immunosorbent assay with purified lipopolysaccharide as the antigen, were removed by adsorption against whole cells of *P. aeruginosa* PAO1 strain H103 (Poole and Hancock, in press).

RESULTS

Phosphate-starvation-induced membrane proteins of members of the *Pseudomonadaceae* and the *Enterobacteriaceae*. Under conditions of phosphate limitation *P. aeruginosa* is derepressed for the synthesis of protein P (11) (Fig. 1, compare lanes 1 and 2), a phosphate-selective, channel-forming outer membrane protein (11). Growth of other pseudomonads as well as members of the family *Enterobacteriaceae* (Table 1) in a phosphate-deficient medium resulted, in many of these strains, in the induction of novel membrane proteins (Fig. 1), many of which existed as the major cell envelope protein. Preliminary studies demonstrated that these were outer membrane proteins (data not shown). The phosphate-starvation inducibility of the PhoE outer membrane proteins of *E. coli* (19) and *S. typhimurium* (2) has been demonstrated previously. In our hands, however, the PhoE protein of *E. coli* comigrated with the OmpF protein of this strain (Fig. 1, lanes 17 and 18), making it necessary to use an OmpF mutant strain (JF700) (Table 1) to demonstrate PhoE induction (Fig. 1, lanes 15 and 16). New membrane proteins were not detected in cell envelopes of *Xanthomonas maltophilia*, *P. acidovorans*, or *Pseudomonas solanacearum* grown in a phosphate-deficient medium, although these strains were derepressed for the synthesis of alkaline phosphatase in this medium (data not shown). *Pseudomonas stutzeri*, which apparently failed to produce a new membrane protein under these circumstances as well, grew extremely poorly in this medium, and *Pseudomonas syringae* did not grow at all, although both of these strains grew quite well in phosphate-sufficient medium.

While most strains expressed a single phosphate-starvation-induced membrane protein band (Fig. 1), *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* each apparently expressed two bands (Fig. 1, lanes 4 and 8, respectively), and *P. cepacia* apparently expressed three bands (Fig. 1, lane 12). With the exception of the 20.5K protein of *P. cepacia* and the 22K protein of *P. fluorescens*, all of the phosphate-starvation-induced membrane proteins were heat modifiable (Table 2). The higher-molecular-weight (non-heat-modified) forms of these proteins were observed as smeared bands in SDS-polyacrylamide gel electrophoretograms. This smearing pattern might be attributed to the association of these proteins with other cell envelope components, such as lipopolysaccharide. To demonstrate the identity of these smears with the monomer (heat-modified) bands described in Fig. 1 and Table 2, we analyzed these bands by the two-dimensional (unheated by heated) gel electrophoretic method of Russell (24). These data (not shown) in all cases except that of *P. cepacia* (see footnotes to Table 2) unambiguously demonstrated that the smeared high-molecular-weight bands were the non-heat-modified (presumably trimeric [1]) forms of the phosphate-starvation-induced proteins described in Table 2. Examination of the solubility, under different conditions, of the non-heat-modified forms of the phosphate-starvation-inducible membrane proteins (Table 2) revealed the existence of two classes of proteins represented by protein P and the major enterobacterial porins (including PhoE).

Immunological cross-reactivity of phosphate-starvation-induced membrane proteins. SDS-polyacrylamide gel electrophoretograms of phosphate-limited cell envelopes were electrophoretically transferred to nitrocellulose and probed

TABLE 2. Properties of the phosphate-starvation-induced membrane proteins

Strain	Apparent mol wt (in thousands) after solubilization at (°C)[a]:		Solubility properties of native oligomers[b]
	88	23	
P. aeruginosa	48[c]	97[c]	A
P. fluorescens	50[c]	102[c]	A
	22	22	ND
P. putida	45.5[c]	97[c]	A
P. chlororaphis	49.5[c]	110[c]	A
	37	ND[d]	—[e]
P. aureofaciens	48[c]	107[c]	A
P. cepacia	37[c]	115[f]	C
	24	115[f]	C
	20.5	20.5	ND
P. pseudomallei	39[c]	104[c]	C
E. coli	37[c]	83[c]	B,C
S. typhimurium	36[c]	82[c]	B,C
K. pneumoniae	36.5[c]	83[c]	B,C
Enterobacter aerogenes	36[c]	85[c]	B,C
Serratia marcescens	37[c]	87[c]	C

[a] Enriched, soluble preparations of the phosphate-starvation-induced proteins were solubilized at 23 or 88°C prior to electrophoresis on SDS-polyacrylamide slab gels. Low-molecular-weight standards (Sigma Chemical Co., St. Louis, Mo.) were coelectrophoresed, and a plot of log molecular weight versus R_f (measured as distance migrated in centimeters) for the standards was derived, from which molecular weights of the phosphate-starvation-induced proteins were determined from their respective R_f values. Because the proteins solubilized at 23°C occurred as smeared bands (see the text) the distance migrated (R_f) was determined for the midpoint of the area of densest staining.

[b] A, Extractable from cell envelopes in 2% (wt/vol) Triton X-100–20 mM Tris hydrochloride (pH 8.0)–0.5 M EDTA; B, extractable from cell envelopes in 2% (wt/vol) Triton X-100–20 mM Tris hydrochloride (pH 8.0) after a 30-min incubation at 37°C in the presence of 1 mg of lysozyme per ml; C, extractable from cell envelopes in 2% (wt/vol) SDS–0.5 M NaCl. ND, Not determined.

[c] Probable cross-reactive protein (in the oligomer form); see the text for a discussion.

[d] The protein solubilized at 23°C did not occur as a smeared band in SDS-polyacrylamide gels. Since the cross-reactive protein in *P. chlororaphis* was identifiable as a smeared band (Fig. 3), this excluded the non-heat-modified form of the 37K protein as the cross-reactive component.

[e] The 37K protein of *P. chlororaphis* was extractable from cell envelopes in 2% (wt/vol) Triton X-100 alone and is probably an inner membrane protein.

[f] The 37K and 24K proteins copurified by all methods tested such that the resultant oligomers at 23°C could not be distinguished but appeared as a high-molecular-weight smear of approximately 115K. As such the identity of the cross-reactive species could not be unambiguously ascertained. Based on the molecular weights of the proteins solubilized at 88°C (heat-modified form), the likely candidate was the 37K protein, whose monomer molecular weight was more typical of porins in general.

with a protein P-trimer-specific (Fig. 2, lane 1; cf. lane 2) polyclonal antiserum. This antiserum was demonstrated to specifically detect protein P trimers in cell envelopes of phosphate-limited *P. aeruginosa* cells (Poole and Hancock, in press). A typical reaction profile of the antiserum against an unheated phosphate-limited (induced for protein P) cell envelope of *P. aeruginosa* can be seen in Fig. 2, lane 4. The smearing pattern of the unheated form of protein P, described above, was also evident in these Western blots.

The protein P-specific antiserum did not interact with any components of the cell envelopes of the organisms studied here when these organisms were grown under phosphate-sufficient conditions (Fig. 3). This suggested that our antiserum was not generally reactive with outer membrane antigens. In contrast, the antiserum reacted with a smeared band corresponding to the non-heat-modified form of the phosphate-starvation-inducible outer membrane proteins of a variety of strains (Fig. 3). In addition, the antiserum reacted with a similar band from the cell envelopes of *E. coli* K-12 strain JF694 which lacks the major porin proteins OmpF and OmpC but is constitutive for the PhoE porin (Fig. 3, lane 25). Solubilization of cell envelopes at 88°C for 10 min, which converted oligomeric proteins to monomers (Table 2), destroyed this reactivity for all proteins (data not shown). This was consistent with the inability of the antiserum to react with purified protein P monomers (Fig. 2, lane 2) or with protein P monomers in heat-treated phosphate-limited cell envelopes (Fig. 2, lane 5). These data excluded the non-heat-modifiable 20.5K and 22K proteins of *P. cepacia* and *P. fluorescens*, respectively, as the cross-reactive species in these strains. A strong cross-reactivity originally seen with *P. cepacia* (Fig. 3, lane 11) was demonstrated to be due, in part, to the presence of alkaline phosphatase associated with the cell envelope (Fig. 3, lane 13). Using a 2% SDS–0.5 M NaCl-soluble preparation of a phosphate-limited *P. cepacia* cell envelope, which contained the phosphate-starvation-induced proteins but lacked alkaline phosphatase, we detected a weak reactivity with the protein P-trimer-specific antiserum (Fig. 3, lane 14).

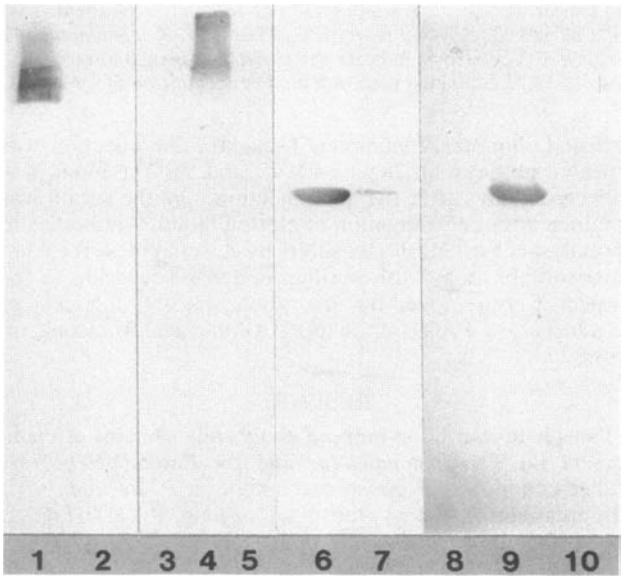

FIG. 2. Interaction of protein P-trimer-specific or -monomer-specific antiserum with Western blots of purified protein P and *P. aeruginosa* PAO1 strain H103 cell envelopes. Cell envelope or purified proteins were electrophoretically transferred from SDS-polyacrylamide gel electrophoretograms to nitrocellulose and incubated with a protein P-trimer-specific (lanes 1 to 5) or -monomer-specific (lanes 6 to 10) antiserum. Antibody binding was detected with an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody (for the trimer-specific antiserum) or an alkaline phophatase-conjugated goat anti-mouse immunoglobulin G antibody (for the monomer-specific antiserum) and a histochemical alkaline phosphatase substrate. Lanes: 1 and 7, purified protein P solubilized at 23°C (trimer form); 2 and 6, purified protein P solubilized at 88°C (monomer form); 3, cell envelope preparation of phosphate-sufficient *P. aeruginosa* solubilized at 23°C; 4 and 10, cell envelope preparation of phosphate-deficient *P. aeruginosa* solubilized at 23°C; 5 and 9, cell envelope preparation of phosphate-deficient *P. aeruginosa* solubilized at 88°C; 8, cell envelope preparation of phophate-sufficient *P. aeruginosa* solubilized at 88°C. A small amount of monomer protein P was detectable in the trimer preparation in lane 7.

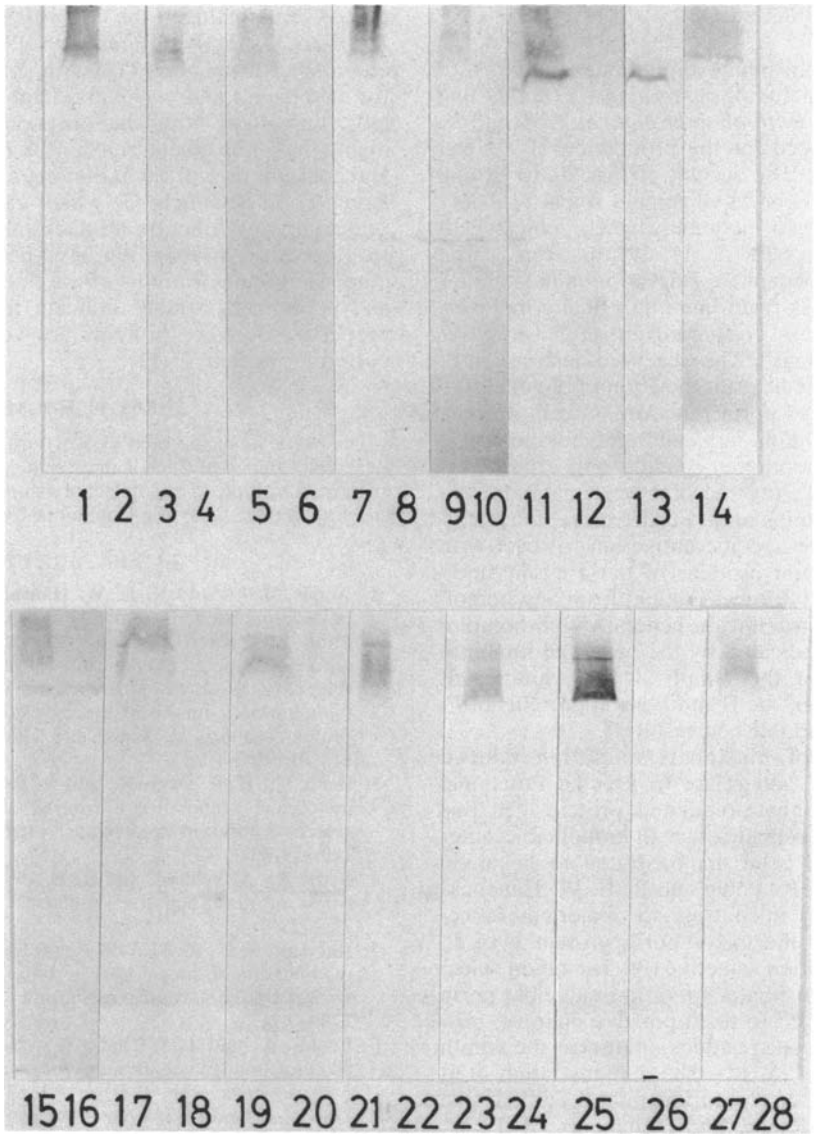

FIG. 3. Interaction of protein P-trimer-specific antiserum with Western blot of cell envelope preparations of different bacteria grown under phosphate-deficient or -sufficient conditions. Except as noted below, odd-numbered lanes contained phosphate-deficient organisms and even-numbered lanes contained phosphate-sufficient organisms. Lanes: 1 and 2, *P. aeruginosa*; 3 and 4, *P. fluorescens*; 5 and 6, *P. putida*; 7 and 8, *P. chlororaphis*; 9 and 10, *P. aureofaciens*; 11 and 12, *P. cepacia*; 13, *P. cepacia*; 14, phosphate-deficient *P. cepacia* cell envelopes solubilized in 2% SDS–0.5 NaCl to inactivate contaminating alkaline phosphatase; 15 and 16, *P. pseudomallei*; 17 and 18, *K. pneumoniae*; 19 and 20, *Enterobacter aerogenes*; 21 and 22, *Serratia marcescens*; 23 and 24, *S. typhimurium*; 25, L-broth-grown *E. coli* K-12 strain JF694; 26, L-broth-grown *E. coli* K-12 strain HMSL74; 27 and 28, *E. coli* K-12 strain JF700. Cell envelopes were solubilized at 23°C prior to electrophoresis in the SDS-polyacrylamide gels, which were subsequently blotted. The blots, with the exception of lane 11, were developed with the protein P-trimer-specific antiserum as described in the legend to Fig. 2. The blot in lane 13 was incubated directly with an alkaline phosphatase histochemical substrate to detect contaminating cell envelope-bound alkaline phosphatase. Similar controls were negative for all other strains shown here.

Immunological cross-reactivity of porin proteins has been demonstrated for the porin monomers of a number of enterobacterial strains (13, 21), consistent with the presence of conserved linear determinants in these proteins. To test whether the cross-reactivity of phosphate-starvation-inducible outer membrane proteins could be attributed to conserved linear determinants, we examined the ability of the monomeric and oligomeric forms of these proteins to react with an antiserum raised against heat-dissociated protein P monomers. The antiserum was demonstrated to react specifically with protein P monomers in heat-denatured cell envelopes of phosphate-limited *P. aeruginosa* (Fig. 2, lane 9) or in preparations purified in detergent (Fig. 2, lane 6) but exhibited no reactivity with the trimer form of the protein (Fig. 2, lanes 7 and 10) or with uninduced (phosphate-sufficient) cell envelopes (Fig. 2, lane 8). This antiserum also failed to react with the phosphate-starvation-inducible outer membrane proteins of the other bacteria described here irrespective of whether these proteins were in the monomer or oligomer forms (data not shown).

DISCUSSION

Under conditions of phosphate limitation, a number of gram-negative bacteria, including *P. aeruginosa* (11), *E. coli* (20), *S. typhimurium* (2), *Enterobacter cloacae* (28), and *K. aerogenes* (25), are induced for the production of a novel outer membrane protein. The results of this study extend this list. These proteins exist as oligomers (most probably trimers) which dissociate to monomers when subjected to temperatures greater than 60°C (2, 11, 29; this study). The demonstration here that phosphate-starvation-induced outer membrane oligomers cross-react immunologically indicates that these proteins express conserved antigenic sites (regions of structural homology). The observed destruction of this cross-reactivity by the heat dissociation of oligomers to monomers, which destroys porin function as well, and the failure of phosphate-starvation-induced monomers to cross-react with a protein P monomer-specific antiserum, suggested that the conserved antigenic sites were maintained in the native, functional proteins only. Furthermore, the inability of the protein P-trimer-specific antiserum to react with the major constitutive porin proteins of these strains indicated that the cross-reactivity was distinct from any homologies relating to poring structure in general. Such homologies do in fact exist, as indicated by the observed immunological cross-reactivity of the OmpF, OmpC, and PhoE porins of *E. coli* (21) and of the F and P porin proteins of *P. aeruginosa* (K. Poole, unpublished results).

The cross-reactivity of phosphate-starvation-induced oligomeric proteins may well relate to specific functional properties of these phosphate-regulated proteins. In this vein, a number of the phosphate-starvation-inducible outer membrane proteins have been demonstrated to be anion selective (2, 3, 11, 20, 28; K. Poole and R. E. W. Hancock, manuscript in preparation); in contrast, the major enterobacterial porin proteins and the major porin protein F of *P. aeruginosa* are weakly cation selective (9). The anion selectivity of two of these phosphate-starvation-inducible porin proteins has been attributed to fixed positive charges, possibly ϵ-amino groups of lysine residues, in or near the mouth of the respective channels (5, 12). The demonstration of at least 14 exposed, trinitrobenzenesulfonate-accessible lysine residues in the protein P and PhoE channels (R. E. W. Hancock, unpublished results) is consistent with this idea. By comparison, the OmpF porin protein contains only five accessible lysine residues (R. E. W. Hancock, unpublished results). It is tempting to hypothesize that exposed lysine residues present in these, and perhaps other, phosphate-regulated porin proteins may be involved in the observed cross-reactivity of phosphate-starvation-inducible outer membrane proteins, perhaps forming part of the conserved antigenic site(s).

Despite the cross-reactivity data described here, phosphate-regulated porin proteins apparently fall into two classes based on the molecular weight of heat-dissociated monomers (45.5K to 50K or 36K to 39K), and the detergent conditions (Triton X-100-Tris-EDTA or SDS-high salt concentration) necessary to release the native proteins from the peptidoglycan (Table 2). Thus, the phosphate-starvation-inducible outer membrane proteins of the *P. fluorescens* branch of the family *Pseudomonadaceae*, including *Pseudomonas putida*, *P. fluorescens*, *Pseudomonas aureofaciens*, and *P. chlororaphis*, were typical of *P. aeruginosa* protein P in these respects, while the analogous proteins of *P. cepacia*, *P. pseudomallei*, and the strains from the family *Enterobacteriaceae* were similar to *E. coli* PhoE porin protein. Interestingly, the channels formed by protein P and PhoE are distinctly different as well. Protein P forms small, anion-specific channels (11) with demonstrated binding sites for anions (12) and phosphate (Hancock and Benz, submitted), consistent with the observed role of protein P in high-affinity phosphate transport in *P. aeruginosa* (Poole and Hancock, in press). Such binding sites are absent from the larger PhoE channels (3) which exhibit only weak anion-selectivity and function in many instances as general diffusion pores (3, 4, 16a). We have purified the cross-reactive outer membrane proteins of the fluorescent pseudomonads, and preliminary studies indicate that the channel-forming properties of these proteins are very similar to those reported for protein P (11).

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