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Cloning of the *Pseudomonas aeruginosa* Outer Membrane Porin Protein P Gene: Evidence for a Linked Region of DNA Homology

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The gene encoding the outer membrane phosphate-selective porin protein P from *Pseudomonas aeruginosa* was cloned into *Escherichia coli*. The protein product was expressed and transported to the outer membrane of an *E. coli phoE* mutant and assembled into functional trimers. Expression of a product of the correct molecular weight was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis, using polyclonal antibodies to protein P monomer and trimer forms. Protein P trimers were partially purified from the *E. coli* clone and shown to form channels with the same conductance as those formed by protein P from *P. aeruginosa*. The location and orientation of the protein P-encoding (*oprP*) gene on the cloned DNA was identified by three methods: (i) mapping the insertion point of transposon Tn*501* in a previously isolated *P. aeruginosa* protein P-deficient mutant; (ii) hybridization of restriction fragments from the cloned DNA to an oligonucleotide pool synthesized on the basis of the amino-terminal protein sequence of protein P; and (iii) fusion of a *PstI* fragment of the cloned DNA to the amino terminus of the β-galactosidase gene of pUC8, producing a fusion protein that contained protein P-antigenic epitopes. Structural analysis of the cloned DNA and *P. aeruginosa* chromosomal DNA revealed the presence of two adjacent *PstI* fragments which cross-hybridized, suggesting a possible gene duplication. The P-related (PR) region hybridized to the oligonucleotide pool described above. When the *PstI* fragment which contained the PR region was fused to the β-galactosidase gene of pUC8, a fusion protein was produced which reacted with a protein P-specific antiserum. However, the restriction endonuclease patterns of the PR region and the *oprP* gene differed significantly beyond the amino-terminal one-third of the two genes.

The high-affinity phosphate uptake system of *Pseudomonas aeruginosa* contains a number of inducible genes which are expressed when the cells are starved of phosphate (8, 22). Many of the components of this system are analogous to those of the *Escherichia coli* high-affinity phosphate-specific transport (PST) system (22, 29). The outer membrane porin, protein P, induced in *P. aeruginosa* differs significantly from the PhoE porin of the *E. coli* PST system (10). The PhoE protein is a typical trimeric porin with large, weakly ion-selective channels (1), although it does appear to be selective for polyphosphates (12). In contrast, protein P trimers form constricted, anion-specific channels which contain a strong phosphate-binding site (2, 6, 8). This binding site is saturable and conducive to kinetic studies. In this regard, protein P is an excellent model for examining structure-function relationships in facilitated diffusion proteins. Protein P is a trimer in its native state and can be dissociated into monomers when heated to temperatures above 60°C (8).

Antigenic studies of porins have provided insights into similarities of structure. For instance, antibodies directed against protein P trimers cross-react with the PhoE porin (24). These antibodies do not react with monomers of either protein P or the PhoE protein (24). This suggests conserved antigenic determinants in native functional trimers. However, antibodies directed against protein P monomers do not react with the PhoE protein (24). In contrast, heat-dissociated porin monomers derived from OmpF, OmpC, and PhoE porins of *E. coli* have been demonstrated to cross-react immunologically (21). In addition, nucleotide analysis of the genes from these three proteins show a 60% homology in the primary protein structures (11, 17, 20, 28). This suggests the possibility of a gene duplication followed by divergence during evolution.

In this paper, we report the cloning of the protein P gene (*oprP*) and the discovery of a homologous region within 1 kilobase pair (kb) of the protein P gene which cross-hybridizes to *oprP* sequences.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in these studies are described in Table 1. *E. coli* strains were maintained on Luria broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl) (14). *P. aeruginosa* strains were maintained on either 1% (wt/vol) Proteose Peptone no. 2 (Difco Laboratories, Detroit, Mich.) or LB containing 0.05% instead of 0.5% (wt/vol) NaCl. MOPS [3-(*N*-morpholino)-propane sulfonate] minimal medium (19) was used with the addition of 660 μM (phosphate-sufficient medium) or 41 μM (phosphate-deficient medium) phosphate. For auxotrophs, these media were supplemented with 50 μg of necessary amino acids per ml and 1 μg of thiamine per ml. Antibiotics were used at the following concentrations: tetracycline, 15 μg/ml; chloramphenicol, 20 μg/ml; and ampicillin, 50 μg/ml. $HgCl_2$ was used at 15 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was used as an indicator of β-galactosidase activity. Media were solidified for use in plates with 2% (wt/vol) agar (Difco).

DNA procedures. Standard recombinant DNA procedures (ligations, restriction digests, transformations, etc.) were as described by Maniatis et al. (14). Southern hybridizations were performed using Zeta-probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.) by the method of Reed and Mann (25). *P. aeruginosa* chromosomal DNA was isolated by treating cells with lysozyme and pronase followed by a series of phenol-chloroform extractions and

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Bacterial strains		
P. aeruginosa PAO		
H103	PAO1 wild type	7
H576	Tn*501* insertion mutant of H103; noninducible for protein P	23
E. coli K-12		
LE 392	F$^-$ *hsdR514* (r_K^- m_K^-) *sup*E*44 supF58 lacY1* or Δ(*lacIZY*)*6 galK2 galT22 metB1 trpR55* λ$^-$	18
CE1194	F$^-$ *thr leu proA2* Δ(*proA-phoE*-gp$^+$) *his thi argE lacY galK xyl rpsL pho-521*	J. Tommassen
JM101	Δ(*lac-pro*) *supE thi* (F′ *traD36 proAB lacI*^{q})	J. Messing
DH5α	ϕ80d*lacZ* ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *sup-44 thi-1* λ$^-$ *gyrA relA1* F$^-$ Δ(*lacZYA-argF*)*U169*	Bethesda Research Laboratories, Gaithersburg, Md.
Plasmids		
pBR325	Cm^r Tc^r Ap^r, ColE1 replicon	3
pUC18	Ap^r, ColE1 replicon	J. Messing
pRJ-1	Ap^r Tc^r Hg^r, 9.0-kb *Pst*I fragment, H576 chromosomal DNA containing Tn*501*, ligated into pBR325	This study
pRS-XP	Ap^r 8.4-kb *Xho*I fragment of PAO chromosomal DNA containing the protein P gene ligated in the *Sal*I site of pUC18	This study
pRSP-01	Ap^r, 700-bp *Hin*dIII fragment of pRS-XP cloned into pUC18	This study
pRSP-02	Ap^r, subclone of the 450-bp *Hin*dIII-*Eco*RI fragment from pRSP-01	This study
pRSP-03	Ap^r, subclone of the 250-bp *Eco*RI-*Hin*dIII fragment from pRSP-01	This study
pRSP-04	Ap^r, subclone of the 500-bp *Hin*dIII-*Pst*I fragment from pRSP-01	This study
pRSP-05	Ap^r, subclone of the 200-bp *Pst*I-*Hin*dIII fragment from pRSP-01	This study
pRSP-06	Ap^r, subclone of the 1.2-kb *Hin*dIII fragment from pRS-XP	This study
pRSP-07	Ap^r, subclone of the 1.6-kb *Eco*RI fragment from pRS-XP	This study
pRSP-08	Ap^r, subclone of the 1.7-kb *Pst*I fragment from pRS-XP	This study
pRSP-09	Ap^r, subclone of the 500-bp *Hin*dIII fragment from pRS-XP	This study
pRSP-10	Ap^r, subclone of the 300-bp *Hin*dIII-*Eco*RI fragment from pRSP-09	This study
pRSP-11	Ap^r, subclone of the 350-bp *Hin*dIII-*Pst*I fragment from pRSP-09	This study
pRSP-12	Ap^r, subclone of the 150-bp *Pst*I-*Hin*dIII fragment from pRSP-09	This study
pRS-13	Ap^r, subclone of the 1.1-kb *Pst*I fragment of pRS-XP into pUC8 creating a gene fusion product	This study
pRS-13R	Ap^r, same as pRS-13 with the insert in the opposite orientation	This study
pRS-8	Ap^r, subclone of the 1.7-kb *Pst*I fragment of pRS-XP into pUC8 creating a gene fusion product	This study
pRS-8R	Ap^r, same as pRS-8 with the insert in the opposite orientation	This study

[a] Abbreviations: Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance; Hg^r, $HgCl_2$ resistance.

ethanol precipitations as described elsewhere (15). DNA was radiolabeled for use as probes by the random hexamer primer method (4).

SDS-polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described previously (9), using a 10% (wt/vol) acrylamide running gel. The Western blot (immunoblot) procedure used was performed as described previously (24). Production of rabbit antibodies against protein P monomer and trimer forms was done as described elsewhere (8, 24). Whole-cell lysate samples, loaded on gels (5 μl per well), were obtained by suspending a pellet from 1.5 ml of a stationary-phase bacterial cell culture in 50 μl of 2% (wt/vol) SDS–4% (vol/vol) 2-mercaptoethanol–10% (vol/vol) glycerol–0.125 M Tris hydrochloride (pH 6.5)–0.001% (wt/vol) bromophenol blue. Samples were heated to 100°C for 10 min before electrophoresis. Outer membrane fractions were solubilized in 0.125 M tris (pH 6.8)–4% (wt/vol) SDS–40 mM EDTA–20% (vol/vol) glycerol–0.001% (wt/vol) bromophenol blue before 30 μg of protein was loaded per well for electrophoresis. Samples were heated for 10 min at 100°C before electrophoresis to observe protein P monomers. No heating was done to samples examined for protein P trimers (8).

Outer membrane preparation. The preparation of Triton X-100 insoluble outer membranes was as described previously (27).

Protein P gene mixed-probe oligonucleotide and hybridization. A collection of synthetic 23-mer oligonucleotides (5′-GACAAGGA$^{G}_{A}$TTC$^{TTC}_{AGG}$TTCAA$^{G}_{A}$CT-3′) was deduced from amino acids 24 to 31 (DKEFSFKL) of the N-terminal sequence of the mature protein P (E. A. Worobec, N. L. Martin, W. McCubbin, C. Kay, G. D. Brayer, and R. E. W. Hancock, Biochim. Biophys. Acta, in press), after taking into consideration the codon bias for *P. aeruginosa* and the degeneracy of the genetic code. The mixed probe consisted of 32 different 23-mer sequences. For use as a hybridization probe, the oligonucleotide was end labeled by using T4 polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, Md.) and [γ-^{32}P]ATP. Digested plasmid DNA was transferred to Zeta-probe nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) as described above and hybridized to the probe by the method of Reed and Mann (25). The filters were hybridized with the labeled oligonucleotide at 46°C for 24 h and washed at 43°C.

Partial purification and functional studies of the cloned protein P product. Strains CE1194 and CE1194(pRS-XP) were grown in phosphate-limiting minimal media containing glucose as the sole carbon source to ensure that the production of the small-channel-forming *E. coli* porin, LamB, was

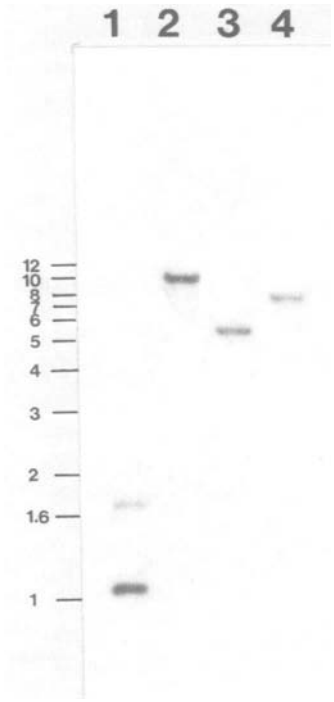

FIG. 1. Southern blot of *P. aeruginosa* chromosomal DNA digested with restriction endonucleases *Pst*I (lane 1), *Pvu*II (lane 2), *Sma*I (lane 3), and *Xho*I (lane 4). The digests were separated by electrophoresis on a 0.7% agarose gel. After the gel was stained with ethidium bromide and photographed, the fragments were transferred to Zeta-probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.) and hybridized to the radiolabeled *Pst*I-*Eco*RI DNA fragments flanking the Tn*501* insertion site in *P. aeruginosa* H576. The migration of the molecular size standards are given in kilobase pairs.

repressed. Partially purified protein P was obtained from strain CE1194(pRS-XP) by extracting Triton X-100 insoluble outer membranes with 2% (wt/vol) SDS in 10 mM Tris (pH 8.0), followed by centrifugation and extraction with 2% (wt/vol) SDS–10 mM Tris (pH 8.0)–20 mM EDTA as described elsewhere (Worobec et al., in press). The resulting supernatant contained protein P trimers along with a low level of the *E. coli* OmpF and OmpC porins. Single-channel conductance studies were carried out as previously described (8), using 1% oxidized cholesterol to form the supporting lipid bilayer. Small samples of the partially purified protein P from strain CE1194(pRS-XP) and the equivalent fraction from strain CE1194 were added to the solution bathing the bilayers (1 M KCl), and the step increases in conductance were recorded. Strain CE1194 preparations were used to determine the conductance of the *E. coli* porins naturally found in this strain (e.g., OmpF and OmpC).

RESULTS

Cloning the protein P gene. *P. aeruginosa* strain H576 is a protein P-deficient strain that was isolated by transposon Tn*501* mutagenesis (23). Chromosomal DNA was isolated from this strain, digested with several restriction endonucleases, and separated on a 0.7% agarose gel. Southern blots of these gels, using radiolabeled Tn*501* DNA as a probe, revealed a single Tn*501* insertion site on the H576 chromosome (data not shown). Since there are no *Pst*I sites in the 7.9-kb Tn*501* DNA sequence (5), the 9.0-kb *Pst*I fragment, from the H576 chromosome, which hybridized to Tn*501* DNA contained 1.1 kb of flanking chromosomal DNA. Therefore, *Pst*I-digested DNA was size fractionated on a 10 to 40% sucrose gradient and the fraction containing the 9.0-kb fragment was isolated, ligated to *Pst*I-digested plasmid pBR325, and transformed into competent *E. coli* LE392 cells. A maximum of 10^5 cells was spread on LB plates containing 15 μg of $HgCl_2$ per ml. Colonies from these plates were screened for ampicillin sensitivity and chloramphenicol resistance. The colonies with this phenotype contained a plasmid harboring Tn*501* on a 9.0-kb *Pst*I fragment. Since there is an *Eco*RI site 15 base pairs (bp) from either terminus of Tn*501* (16), the *Pst*I-*Eco*RI fragments (520 and 660 bp) flanking the insertion element were isolated, pooled, and radiolabeled for use as a protein P gene probe.

P. aeruginosa PAO1 chromosomal DNA digested with *Pst*I, *Xho*I, and *Sma*I had fragments of 1.2, 8.4, and 6.0 kb, respectively, which hybridized to the protein P probes. The *Pst*I digest also showed a weaker hybridizing fragment at 1.7 kb (Fig. 1, lane 1). *Xho*I-digested PAO1 DNA was size fractionated, and the fractions containing the 8.4-kb fragment were isolated, ligated into *Sal*I-cut pUC18, transformed into *E. coli* LE392, and plated on LB plates which contained ampicillin and X-Gal. White colonies were picked onto fresh plates and screened by colony hybridization to the radiolabeled protein P probe. Plasmid pRS-XP (Fig. 2) was isolated from a positively hybridizing colony.

Expression of protein P in *E. coli*. Plasmid pRS-XP was transformed into the *E. coli phoE* mutant CE1194 and grown under phosphate-deficient conditions. Western blots of whole-cell lysates revealed the production of protein P when the cells were grown under conditions that derepress the PST system in *E. coli* (R. J. Siehnel, E. A. Worobec, and R. E. W. Hancock, Mol. Microbiol., in press). Outer mem-

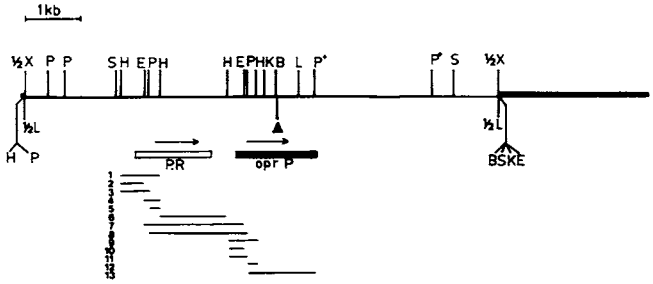

FIG. 2. Restriction map of pRS-XP. The 8.4-kb *Xho*I fragment of *P. aeruginosa* H103 (thin line) was cloned to the *Sal*I site of pUC18 (thick line). The triangle represents the site of the Tn*501* insertion in *P. aeruginosa* H576. The solid bar below the map represents the size and location of *oprP*. The open bar represents the location of the PR region (see Results). The arrows denote the translational direction of the two regions. The thin lines below the map represent the location of DNA subcloned into pUC18, as described in Table 1 and referred to in the legend to Fig. 4. Lines: 1, pRSP-01; 2, pRSP-02; 3, pRSP-03; 4, pRSP-04; 5, pRSP-05; 6, pRSP-06; 7, pRSP-07; 8, pRSP-08; 9, pRSP-09; 10, pRSP-10; 11, pRSP-11; 12, pRSP-12. Region 13 was subcloned into pUC8 in both orientations in pRS-13 (same orientation as the fragment in plasmid pRS-XP) and pRS-13R. Region 8 was also subcloned into pUC8 in pRS-8 (same orientation as the fragment in the plasmid pRS-XP) and pRS-8R. The restriction sites are given by one-letter codes as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; L, *Sal*I; P, *Pst*I; S, *Sma*I; X, *Xho*I. Restriction sites identified below the map originated from vector sequences. There were two further unmapped *Pst*I sites located between the two *Pst*I sites marked with crosses, generating *Pst*I fragments of 0.35, 0.70, and 1.05 kb.

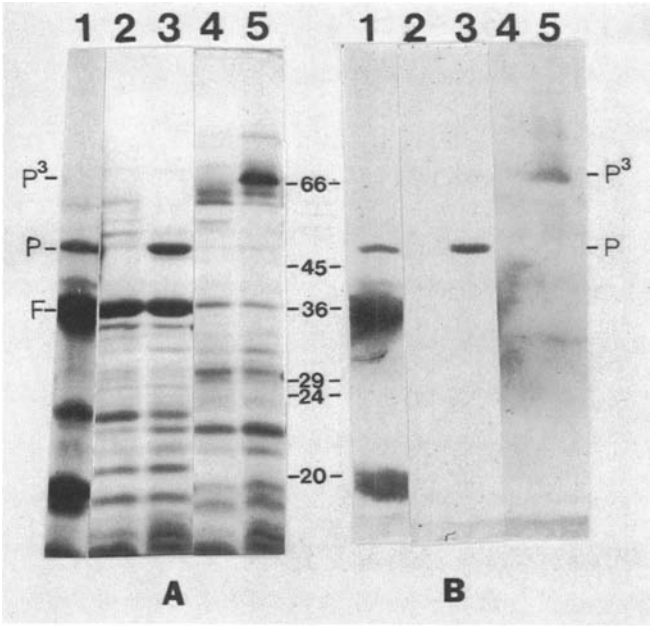

FIG. 3. (A) Coomassie blue-stained 10% SDS-polyacrylamide gel of extracts from *E. coli* CE1194 and CE1194 with plasmid pRS-XP. Extracts were heated to 100°C for 10 minutes before being loaded to observe protein P monomers and were kept at room temperature to observe protein P trimers (8). Lanes: 1, outer membrane preparation from phosphate-starved *P. aeruginosa* H234 heated to 100°C before being loaded; 2, outer membrane preparation from CE1194 heated to 100°C before being loaded; 3, outer membrane preparation from CE1194(pRS-XP) heated to 100°C before being loaded; 4, outer membrane preparation from CE1194, unheated; 5, outer membrane preparation from CE1194(pRS-XP), unheated. (B) Western blots of a gel identical to that in panel A. Lanes 1 to 3 were reacted with rabbit polyclonal serum directed against protein P monomer. Lanes 4 and 5 were reacted with rabbit polyclonal antiserum directed against protein P trimers. P, Location of protein P monomers; P^3, location of lipopolysaccharide-associated protein P trimers; F, location of protein F in lane 1 and OmpF and C proteins in lanes 2 and 3. Migration of molecular mass markers is indicated in kilodaltons.

branes were isolated, and their proteins were separated by SDS-polyacrylamide gel electrophoresis. A band corresponding to protein P monomer was present in the Triton X-100-insoluble outer membranes of strain CE1194(pRS-XP) when heated to 100°C for 10 min (Fig. 3A, lane 3), as confirmed by immunoblots with anti-protein P monomer (Fig. 3B, lane 3). Bands corresponding to protein P trimer were seen when the CE1194(pRS-XP) outer membrane preparation was not heated before electrophoresis (Fig. 3A, lane 5). Western blots with rabbit anti-protein P trimer (24) confirmed the presence of protein P trimers in the cell envelope of *E. coli* CE1194 that contained plasmid pRS-XP (Fig. 3B, lane 5).

Unfortunately, because of the similar physical properties of protein P and the OmpF and C porins of *E. coli*, we were unable to purify, free of contaminating *E. coli* porins, the protein P produced from plasmid pRS-XP. Nevertheless, the substantial differences (1, 2) in the single-channel conductances in 1MKC1 of protein P (0.28 nS) and the *E. coli* porins (1.7 to 1.9 nS) allowed us to clearly differentiate these two classes of channels by model-membrane black-lipid bilayer studies. Analogous preparations from the plasmid-free strain CE1194 and strain CE1194(pRS-XP) containing the cloned protein P gene both formed channels typical of the OmpF and C porins. However, only strain CE1194(pRS-XP) contained channels smaller than 0.6 nS at a frequency of approximately 50% of single-channel conductances observed. For this smaller population, the average single-channel conductance was 0.28 nS, as observed previously for protein P from *P. aeruginosa* (9). This provides powerful evidence that the protein P trimers formed in *E. coli* CE1194(pRS-XP) were capable of reconstituting functional channels.

Mapping of protein P (*oprP*) gene. The approximate location of the protein P gene was determined by mapping the site of insertion of Tn*501* in the clone derived from the protein P-deficient Tn*501* insertion mutant *P. aeruginosa* H576 (indicated by a solid triangle on the restriction map of plasmid pRS-XP; Fig. 2). The region encoding the amino terminus of protein P was demonstrated by hybridization experiments with an oligonucleotide pool that was synthesized on the basis of the known sequence of amino acids 24 to 31 of the mature protein P. This oligonucleotide pool hybridized to subclones 8, 9, and 11 of plasmid pRS-XP (Fig. 2) but not to subclones 6, 7, 10, 12, or 13 (Fig. 2). Since subclones terminating at a given *Eco*RI site (e.g., subclones 7 and 10) failed to hybridize with the probe pool, whereas subclones overlapping this site hybridized (e.g., subclones 9 and 11), this placed the hybridization site at or near this

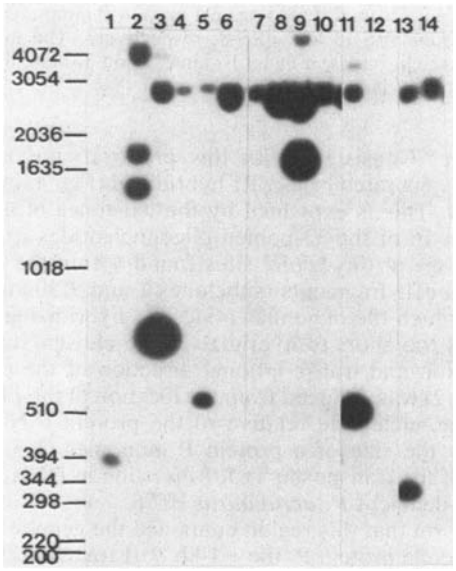

FIG. 4. Southern blot of DNA from plasmid pRS-XP and its derivative subclones. Plasmids with subcloned DNA were isolated from *E. coli* DH5α and digested with restriction endonucleases that precisely separated insert DNA from vector DNA (see Fig. 2 for a description of the inserts of these subclones). The insert DNA fragments were separated by agarose gel electrophoresis. After the gels were stained with ethidium bromide and photographed, the fragments were transferred to Zeta-probe nylon membrane (Bio-Rad Laboratories, Richmond, Calif.) and hybridized to radiolabeled oligonucleotides. See Fig. 2 and Table 1 for a description of plasmids. Lanes: 1, *Hin*fI digest of pBR322; 2, *Pst*I digest of pRS-XP; 3, pRSP-01; 4, pRSP-02; 5, pRSP-03; 6, pRSP-07; 7, pRSP-04; 8, pRSP-06; 9, pRSP-08; 10, pRSP-05; 11, pRSP-09; 12, pRSP-10; 13, pRSP-11; 14, pRSP-12. The migration of molecular size standards is shown in base pairs. One of the oligonucleotides hybridized to a 394-bp *Hin*fI fragment of pBR322 (lane 1) that is also found in pUC18 DNA. Therefore, any bands appearing above the 2,036-bp molecular size marker are due to hybridization to vector DNA.

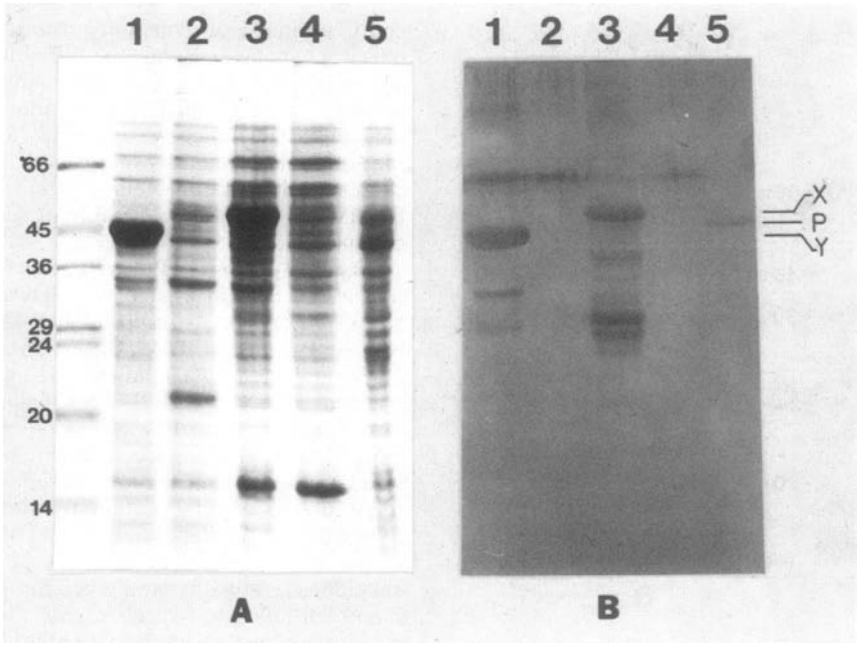

FIG. 5. (A) Coomassie blue-stained 10% SDS-polyacrylamide gel of whole-cell extracts of *E. coli* strains. Lanes: 1, JM101(pRS-8); 2, JM101(pRS-8R); 3, JM101(pRS-13); 4, JM101(pRS-13R); 5, CE1194(pRS-XP). Cells were grown to saturation overnight, pelleted, added to lysis buffer, and heated to 100°C for 10 min before being loaded. (B) Western blots of a gel identical to that in panel A. The blot was reacted with rabbit antiserum directed against protein P monomers. P, Location of protein P monomers; X and Y, Locations of the fusion proteins produced from pRS-13 and pRS-8, respectively. The migration of the molecular mass markers is indicated in kilodaltons. The lower-molecular-weight bands in panel B, lanes 1 and 3, most likely represent breakdown products of the fusion proteins Y and X since they are absent in control lanes 2 and 4.

*Eco*RI site. Consistent with this proposal, no restriction fragments generated by *Eco*RI hybridized (Fig. 4, lanes 4, 6, 7, and 12). This is explained by the existence of an *Eco*RI site within 16 of the 32 pooled oligonucleotides used, such that cleavage at the *Eco*RI sites found within the 500- and 700-bp *Hin*dIII fragments (subclones 1 and 9) disrupted the 23 bp to which the oligonucleotide was hybridizing, leaving sequences too short to hybridize at the chosen stringency. The location and transcriptional direction of the protein P gene (Fig. 2) was deduced from the location of the *Eco*RI site in the oligonucleotide relative to the protein P N-terminal sequence, the size of a protein P monomer (13), and the location of the transposon Tn*501* insertion in DNA from the protein P-deficient *P. aeruginosa* H576.

To confirm that this region contained the genetic information to encode protein P, the 1.1-kb *Pst*I fragment (fragment 13 of Fig. 2) was fused to the β-galactosidase promoter in all three reading frames, using the *Pst*I-cleaved translational fusion vectors pUC8, pUC9, and pUC18. The pUC8 derivative plasmid pRS-13 expressed a 49-kilodalton protein (Fig. 5A, lane 3) which reacted with antibodies directed to protein P monomers (Fig. 5B, lane 3). The somewhat larger mass than the native protein P (47 kilodaltons) was presumably due to the truncation of the gene at the carboxy-terminal *Pst*I site, resulting in translation readthrough into pUC8 sequences.

Evidence for a linked region of homology. A region upstream of the *oprP* gene, called PR (for P related), showed evidence of homology to this gene by three separate criteria. Southern blots of complete *Pst*I digests of *P. aeruginosa* PAO1 chromosomal DNA and of plasmid pRS-XP DNA showed two *Pst*I-generated restriction fragments which hybridized with the probe derived from the sequences flanking the transposon Tn*501* in the protein P-deficient strain H576 (Fig. 1, lane 1). These were the 1.1-kb *Pst*I fragment from the *oprP* gene (fragment 13 in Fig. 2) and an adjacent 1.7-kb *Pst*I fragment from the PR region (fragment 8 in Fig. 2). The observation of both fragments in complete chromosomal digests demonstrated that the repeated regions were not an artifact of cloning. In addition, the restriction map of plasmid pRS-XP revealed that one end of each of these two adjacent *Pst*I fragments contained three identically spaced restriction sites in the order *Eco*RI-*Pst*I-*Hin*dIII (Fig. 2). Nevertheless, the remainder of the restriction patterns of these *Pst*I fragments differed, demonstrating that the 1.7-kb fragment was not an incomplete digestion product from which the 1.1-kb fragment arose.

The *Eco*RI site of the above-described *Eco*RI-*Pst*I-*Hin*dIII repeat was contained within the sequence of the *oprP* gene which hybridized to the oligonucleotide pool deduced from the N-terminal amino acid sequence of protein P (see above). To see whether the analogous sites from the PR region were homologous, we examined hybridization to the enzymatically digested parent plasmid pRS-XP and to a variety of derivative subclones. Two hybridizing fragments were observed after *Pst*I digestion (Fig. 4, lane 2) of the parent plasmid pRS-XP. Two *Hin*dIII fragments also hybridized (Fig. 4, lanes 3 and 11), but no restriction fragments generated by *Eco*RI were capable of hybridizing (Fig. 4, lanes 4, 6, 7, and 11). Subclones 1 and 3 (see Fig. 2 for a description of the inserts) hybridized (Fig. 4, lanes 3 and 5), whereas those subclones terminating at the *Eco*RI site of the repeat region, subclones 2 and 4, failed to hybridize (Fig. 4, lanes 4 and 7). These data are consistent with the conclusion that the *Eco*RI site of the *Eco*RI-*Pst*I-*Hin*dIII repeat from the PR region was part of the sequence hybridizing to the oligonucleotide pool.

To further examine homology of the PR region with the

oprP gene, the 1.7-kb *Pst*I fragment 8 (Fig. 2) was cloned into pUC8 to form plasmid pRS-8. A fusion protein (Fig. 5A, lane 1), which reacted with antiserum specific for protein P monomers (Fig. 5B, lane 1), was synthesized in cells containing plasmid pRS-8. However, the fusion gene product of plasmid pRS-8 was of a lower molecular weight (i.e., 44,500) than the fusion protein derived from the 1.1-kb *Pst*I insert of the *oprP* gene (49,000; Fig. 5, lane 3).

DISCUSSION

The gene for the phosphate-regulated outer membrane porin, protein P, of *P. aeruginosa* was cloned, using a probe derived from the Tn*501*-flanking sequences of the Tn*501*-induced protein P-deficient *P. aeruginosa* H576. The hybridization of this probe to *Pst*I-cut *P. aeruginosa* chromosomal DNA revealed two unique fragments which showed homology to the Tn*501*-flanking sequences of H576. Since the probe had a *Pst*I terminus, this suggested the possibility of a duplication within the genome. An 8.4-kb *Xho*I fragment from *P. aeruginosa* H103, which contained the protein P gene, was isolated. Analysis of this clone revealed both *Pst*I fragments to be found in tandem on the chromosome. Both *Pst*I fragments were inserted into the translational fusion vector pUC8 to produce polypeptides which reacted with protein P-specific antiserum. Nevertheless, the protein P structural gene *oprP* could be discerned by mapping the site of insertion of the Tn*501* from the above-mentioned protein P-deficient mutant. Hybridization experiments with oligonucleotides, whose sequences were deduced from the amino-terminal amino acid sequence of protein P, revealed the presence of two regions of the plasmid pRS-XP, each containing an *Eco*RI site, which corresponded to protein P amino-terminal sequences. The regions immediately downstream from these two *Eco*RI sites, constituting about one-third of the *oprP* gene and PR region, were found to have an identical restriction pattern (Fig. 1). These data provided strong evidence that the adjacent PR DNA region is homologous to the *oprP* gene. However, although the production of a fusion protein in *E. coli* JM101(pRS-8) is consistent with the suggestion that the PR region contains an open reading frame, we do not know whether this region codes for and expresses a gene product. This question and the degree of homology to *oprP* is currently being addressed by promoter switching and DNA sequencing.

The protein P produced by plasmid pRS-XP appears in the outer membrane as a trimer (Fig. 3) which forms typical P-like channels in lipid bilayer membranes. Thus, it appears that *E. coli* can efficiently process and transport protein P to the outer membrane, where the protein is assembled into the proper quaternary structure necessary for channel formation. This will allow us the flexibility of using *E. coli* as a host for gene manipulation studies of structure-function relationships of protein P.

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