

Molecular Cloning, Characterization, and Regulation of a *Pseudomonas pickettii* PKO1 Gene Encoding Phenol Hydroxylase and Expression of the Gene in *Pseudomonas aeruginosa* PAO1c

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A 26-kilobase *Bam*HI restriction endonuclease DNA fragment was cloned from *Pseudomonas pickettii* PKO1, a strain isolated from a soil microcosm that had been amended with benzene, toluene, and xylene. This DNA fragment, cloned into vector plasmid pRO1727 and designated pRO1957, allowed *Pseudomonas aeruginosa* PAO1c to grow on phenol as the sole source of carbon. Physical and functional restriction endonuclease maps have been derived for the cloned DNA fragment. Two DNA fragments carried in *trans* and derived from subclones of pRO1957 show phenol hydroxylase activity in cell extracts of *P. aeruginosa*. Deletion and subcloning analyses of these fragments indicated that the gene encoding phenol hydroxylase is positively regulated. Phenol and *m*-cresol were shown to be inducers of the enzyme. *o*-Cresol and *p*-cresol did not induce enzymatic activity but could be metabolized by cells that had been previously exposed to phenol or *m*-cresol; moreover, the enzyme exhibited a rather broad substrate specificity and was sensitive to thiol-inhibiting reagents. A novel polypeptide with an estimated molecular mass of 80,000 daltons was detected in extracts of phenol-induced cells of *P. aeruginosa* carrying plasmid pRO1959.

Phenol and structurally related compounds, such as cresols, alkylphenols, xylenols, and catechol, are formed as by-products in many industrial processes (9) and are listed by the U.S. Environmental Protection Agency (20) as high-priority pollutants. A number of microorganisms have been found to degrade phenol (1, 2, 18, 21, 28, 29, 33), and it has been shown that the first step in the catabolism of phenol in oxygenated environments is its hydroxylation to catechol, a reaction catalyzed by phenol hydroxylase (EC 1.14.13.7). Phenol hydroxylase has been purified and extensively characterized from the mesophilic yeast *Trichosporon cutaneum* (10, 11, 28), and a thermostable enzyme has been partially purified from *Bacillus stearothermophilus* (15). Studies on regulation of the enzymes involved in catabolism of phenol and the *meta* cleavage of catechol produced from phenol have been done on *Pseudomonas putida* U (3, 8, 40); however, phenol hydroxylase has not been purified from this *Pseudomonas* strain. Recently, plasmid-encoded genes for a multicomponent phenol hydroxylase have been characterized from *Pseudomonas* sp. strain CF600 (35), and a multi-plasmid system which determines the degradation of phenol has been identified in *Pseudomonas* sp. strain EST1001 (21).

We have recently isolated a strain of *Pseudomonas pickettii*, designated strain PKO1, from a soil microcosm that had been amended with benzene, toluene, and *p*-xylene (13). *P. pickettii* PKO1 utilized phenol as the sole source of carbon and energy, and phenol-induced cells contained significant levels of phenol hydroxylase activity, both in washed cell suspensions and in cell extracts.

We report here on the molecular cloning of the structural gene encoding phenol hydroxylase from *P. pickettii* PKO1, the expression of this gene in *Pseudomonas aeruginosa* PAO1c, and the mode of regulation of the cloned structural gene encoding phenol hydroxylase.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are described in Table 1. Plasmid pRO1733 was derived by cloning the *Hind*III A fragment from the TOL plasmid pWW0 into the unique *Hind*III site in vector plasmid pRO1614. It had been previously shown (19) that the *xylE* gene, which encodes catechol 2,3-dioxygenase, was located on the *Hind*III A fragment of pWW0. Plasmid pRO1940 was derived by deletion of the *Bam*HI C and E fragments and the *Xho*I E fragment from pRO1733. Plasmid pRO2343 contains the same construction as in plasmid pRO1940 except that the DNA insert is in vector plasmid pRO2321.

Media and growth conditions. Minimal medium of Stanier et al. (MMO; 36) or of Vogel and Bonner (VBG; 37) and complex plate count medium (TN; 31) were prepared as described elsewhere. Tetracycline, carbenicillin (or ticarcillin), trimethoprim, streptomycin, or gentamicin was used in selective media at 50, 500 (250 for ticarcillin), 600, 250, or 5 μ g/ml, respectively. When grown for enzyme assays, bacteria were cultured in 100 ml of MMO medium, with aeration, supplemented with carbon substrates to a final concentration of 10 mM. Phenol or catechol was added to MMO medium to a final concentration of 5 mM, and Casamino Acids (Difco Laboratories, Detroit, Mich.), when used as a supplemental carbon source, were added to a final concentration of 0.3%. *P. aeruginosa* was grown at 37°C. *P. pickettii* was grown at 30°C.

Genetic techniques. Isolation of chromosomal and plasmid DNA, restriction endonuclease digestion, molecular cloning, and transformations were done as described previously (30).

Measurement of enzyme activity. Cells were grown for enzyme assays to a density that gave an apparent A_{425} of 1.0 to 1.5 (Spectronic 21 spectrophotometer; Bausch & Lomb, Inc., Rochester, N.Y.), and were harvested by centrifugation at 10,000 \times *g* for 15 min. The cell pellets were washed twice in 50 mM sodium phosphate buffer (pH 7.6) containing 1 mM beta-mercaptoethanol, 0.1 mM EDTA, and 1 μ M

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant markers ^a	Reference or derivation
Strains		
<i>Pseudomonas aeruginosa</i>		
PAO1c	Prototroph	16
PAO1.93	<i>catA</i>	22
<i>Pseudomonas pickettii</i>		
PKO1	Prototroph, Phl ⁺	13
PKO2	Prototroph	ATCC 27511
Plasmids		
pRO1614	Cb ^r Tc ^r , cloning vector	30
pRO1727	Cb ^r Tc ^r , cloning vector	6
pRO1769	Sm ^r Gm ^r , cloning vector	7
pRO2317	Cb ^r Tc ^r , cloning vector	41
pRO2321	Tp ^r Tc ^r , cloning vector	41
pRO1733	Cb ^r <i>xylS xylDLEGFJKIH</i>	38
pRO1940	Cb ^r <i>xylS xylE</i>	38
pRO1957	Cb ^r Phl ⁺	26-kb <i>Bam</i> HI fragment from <i>P. pickettii</i> PKO1 cloned into pRO1727
pRO1958	Cb ^r Phl ⁻	<i>Hind</i> III deletion of pRO1957
pRO1959	Cb ^r Phl ⁺	Partial <i>Hind</i> III deletion of pRO1957
pRO1960	Cb ^r Phl ⁻	<i>Bgl</i> III deletion of pRO1957
pRO1963	Cb ^r Phl ⁻	<i>Xho</i> I deletion of pRO1959
pRO1964	Cb ^r Phl ⁻	<i>Bgl</i> III- <i>Bam</i> HI digest of pRO1959, religated
pRO1965	Cb ^r Phl ⁻	<i>Bcl</i> I deletion of pRO1959
pRO1966	Cb ^r Phl ⁻	<i>Cla</i> I deletion of pRO1959
pRO1981	Gm ^r Phl ⁻	<i>Eco</i> RI digest of pRO1960, cloned into the <i>Eco</i> RI site of pRO1769
pRO1982	Gm ^r Phl ⁻	<i>Eco</i> RI digest of pRO1960, cloned into the <i>Eco</i> RI site of pRO1769
pRO1983	Gm ^r Phl ⁻	<i>Eco</i> RI digest of pRO1960, cloned into the <i>Eco</i> RI site of pRO1769
pRO2339	Cb ^r Phl ⁻	<i>Xho</i> I digest of pRO1959, cloned into the <i>Sal</i> I site of pRO2317
pRO2342	Tp ^r Phl ⁻	<i>Hind</i> III digest of pRO1959, cloned into the <i>Hind</i> III site of pRO2321
pRO2343	Tp ^r <i>xylS xylE</i>	<i>Hind</i> III- <i>Bam</i> HI digest of pRO1940, cloned into <i>Hind</i> III- <i>Bam</i> HI-cleaved pRO2321

^a Abbreviations: Cb^r, Tc^r, Sm^r, Gm^r, Tp^r, Resistance to carbenicillin (or ticarcillin), tetracycline, streptomycin, gentamicin, trimethoprim, respectively. Other abbreviations as in text.

flavin adenine dinucleotide, and the cells were disrupted sonically by four 15-s 200-W bursts with a Braun-Sonic 1510 apparatus. Cellular debris was removed by centrifugation at 100,000 × *g* at 5°C for 1 h, and the clear supernatant solution was used immediately for enzyme assays. Phenol hydroxylase activity was assayed by measuring the decrease in A₃₄₀, using NADPH as the cosubstrate (28). The reaction was performed at 30°C in 1.0-ml quartz cuvettes with 1-cm light path. The final volume of 1.0 ml contained 865 μl of 50 mM sodium phosphate buffer (pH 7.6), 30 μl of 10 mM NADPH, 5 μl of 10 mM phenol, and 100 μl of appropriately diluted cell extract. One unit of enzyme activity is defined as the amount of enzyme which, in the presence of phenol, causes the oxidation of 1 μmol of NADPH per min.

Protein was determined by the method of Bradford (5) with bovine serum albumin as the standard. Enzyme specific activities are reported as micromoles of substrate or cosubstrate utilized per minute per milligram of protein. UV absorbance spectra were measured on a Shimadzu UV-160 spectrophotometer.

Electrophoretic methods. Soluble cellular proteins from 100,000 × *g*-cleared supernatant solutions of cell extracts were separated electrophoretically on sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli (24). Samples were boiled for 3 min in solubilization buffer (5% beta-mercaptoethanol, 2% SDS, 0.44 mM phenylmethylsulfonyl fluoride, 10% glycerol, 0.04% bromophenol blue in 62.5 mM Tris, pH 6.8). Gels were run for 30 min at 25 mA through a 4% acrylamide stacking gel and for a further 2 h at 20 mA through either a 10 or 7% acrylamide separating gel. Protein standards used for molecular mass estimation, and their approximate molecular masses in kilodaltons, were

myosin, 205; beta-galactosidase, 116; phosphorylase *b*, 97.4; bovine albumin, 66; ovalbumin, 45; glyceraldehyde-3-phosphate dehydrogenase, 36; carbonic anhydrase, 29. To visualize proteins, we stained the gels with Coomassie blue.

Analytical methods. Products obtained from incubation of aromatic substrates with cell extracts were analyzed by reverse-phase high-performance liquid chromatography (HPLC). Reaction mixtures were prepared with extracts of cells of *P. aeruginosa* PAO1.93(pRO1959) grown in phenol plus Casamino Acids as described above. Each reaction mixture contained the following (per milliliter): sodium phosphate (pH 7.6), 50 μmol; NADPH, 30 μmol; cell extract (9.4 μg of protein), 10 μl; and aromatic substrate, 5 μmol. A parallel reaction mixture that contained extract of cells of *P. aeruginosa* PAO1.93, which did not carry pRO1959, grown in phenol plus Casamino Acids served as a control. The reaction mixtures were incubated on a rotary shaker at 30°C and the reactions were stopped after 1 h by the addition of 3 volumes of HPLC-grade methanol to precipitate proteins. After a brief centrifugation, 200-μl samples were analyzed for product formation by HPLC (Shimadzu LC-6A pumps and SCL-6B controller; PhaseSep H4726 column [4.6 by 250 mm] filled with Spherisorb ODS2 [diameter, 5 μm] preceded by a Whatman CSKI guard column [6.5 by 65 mm]). The mobile phase was acetic acid-water (ratio by volume, 1:99), the flow rate was 1 ml/min, and analyses were performed with a Shimadzu CR501 Chromatopac computing integrator. Metabolites were identified by comparison of their retention times with those of pure substances.

Chemicals and reagents. All the chemicals, enzymes, and reagents used in these studies were of the highest purity commercially available. Phenol, cresols, catechol, 4-methyl-

catechol, 2-chlorophenol, 3-chlorophenol, 2-chloro-5-methylphenol, 4-chloro-3-methylphenol, 3,4-dimethylphenol, 2,3-dimethylphenol, 2,4-dimethylphenol, 2,5-dimethylphenol, 2,6-dimethylphenol, 3,5-dimethylphenol, *m*-hydroxybenzoate, hydroquinone, pyrogallol, and 1,2,4-benzenetriol were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). 3-Methylcatechol was purchased from Pfaltz and Bauer, Inc. (Waterbury, Conn.). Resorcinol, guaiacol (*o*-methoxyphenol), 2,4-dichlorophenol, and *p*-hydroxybenzoate were from Sigma Chemical Co. (St. Louis, Mo.). Salicylate was purchased from Mallinckrodt Chemical Works (St. Louis, Mo.). 4-Chlorocatechol was obtained from Helix Biotech Ltd. (Richmond, British Columbia, Canada). 3-Chlorocatechol was prepared enzymatically from 2-chlorophenol utilizing an extract of cells of *P. aeruginosa* PAO1c that produced chlorophenol hydroxylase from the cloned *tfdB* gene from plasmid pJP4 (23; B. Kaphammer and R. H. Olsen, unpublished data).

Enzymes and reagents used for DNA manipulations were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used as suggested by the supplier. Disodium ticarcillin was from Beecham Laboratories (Bristol, Tenn.). Tetracycline, trimethoprim, and protein molecular weight markers were obtained from Sigma. Streptomycin and disodium carbenicillin were obtained from Pfizer, Inc. (New York, N.Y.), and gentamicin was from Elkins-Sinn, Inc. (Cherry Hill, N.J.). Vitamin-free Casamino Acids, as well as all other bacteriological medium components, were purchased from Difco.

RESULTS

Strain description. The morphological, cultural, and metabolic characteristics of strain PKO1 are identical in all respects with those of the type strain of *P. pickettii* (ATCC 27511), described by Ralston et al. (32) and designated strain PKO2 in our laboratory, with the exception that strain PKO1 can utilize *m*-cresol and phenol.

Cloning of chromosomal DNA fragment from *P. pickettii* PKO1 carrying genes required for growth on phenol. A *Bam*HI gene bank of genomic DNA from *P. pickettii* PKO1 was constructed in *P. aeruginosa* PAO1c by using vector plasmid pRO1727 according to previously described procedures (30). Gene bank DNA was used to transform cells of *P. aeruginosa* PAO1c. Transformants were initially selected on TN medium containing carbenicillin and were then washed onto minimal phenol medium. Phenol-positive transformants were screened for plasmid content by a rapid alkaline lysis procedure. Results of such a screening revealed that phenol-positive transformants contained a recombinant plasmid that was larger than the pRO1727 cloning vector. After purification of this plasmid DNA in a cesium chloride-ethidium bromide gradient, restriction digest analysis demonstrated that the recombinant plasmid, designated pRO1957, contained a 26-kilobase (kb) *Bam*HI insert. Plasmid pRO1957 was mapped with restriction endonucleases, and the results are shown in Fig. 1.

Subcloning and localization of *phlA*, the gene encoding phenol hydroxylase. To further localize the *phlA* gene on plasmid pRO1957, we made a series of *Hind*III and *Bgl*II deletions. Plasmid pRO1959, which contains a 14.5-kb *Hind*III-*Bam*HI fragment of pRO1957 DNA (Fig. 1), allowed growth on minimal phenol medium when carried in *P. aeruginosa* PAO1c. However, plasmids pRO1958 and pRO1960 did not allow growth on phenol. Further deletion

and subcloning of plasmid pRO1959 resulted in plasmids which did not allow *P. aeruginosa* PAO1c to grow on phenol (Fig. 1).

Previous work on phenol catabolism in *P. putida* U (40) suggested that this structural gene for phenol hydroxylase is under positive transcriptional control. Thus, by analogy here, DNA fragments carrying only the *phlA* structural gene, in the absence of an activator, might be expected to show only a basal level of phenol hydroxylase, insufficient to allow cells to grow on phenol minimal medium. To determine which of the subclones of pRO1959 carried the *phlA* structural gene, we placed each subclone in *trans* with the *xylE* gene of plasmid pWW0, which encodes a catechol 2,3-dioxygenase, borne on a compatible plasmid vector. Cells of *P. aeruginosa* PAO1c carrying these double constructs and grown on minimal glucose plus phenol medium were inspected for yellow color, indicating production of 2-hydroxy-muconate semialdehyde from catechol. Results from such an experiment (Fig. 1) indicated that plasmids pRO1963, pRO2342, and pRO1965 carry the *phlA* gene, reflecting the production of muconate semialdehyde by the *xylE* gene product, catechol 2,3-dioxygenase. The smallest of these plasmids, pRO2342, contains only the internal *Hind*III fragment of the parental clone, pRO1957.

Localization of *phlR*, a gene necessary for transcription of *phlA*. Data described in the foregoing section indicated that the phenol hydroxylase structural gene carried on plasmid pRO2342 was insufficient for growth of *P. aeruginosa* PAO1c on phenol. When cells of *P. aeruginosa* PAO1c carrying plasmid pRO2342 were transformed with each of the deletants of pRO1959 listed in Fig. 1, only plasmid pRO1960 allowed growth on phenol when in *trans* with pRO2342. Plasmid pRO1960 contains a DNA fragment that partly overlaps the DNA insert in pRO2342. Plasmid pRO1958, which contains the *Hind*III-*Bam*HI fragment immediately adjacent to the internal *Hind*III fragment of pRO1957, did not allow growth on phenol when in *trans* with pRO2342, suggesting that the *phlR* gene spans the *Hind*III site at map coordinate 14.75 kb (Fig. 1). Similarly, plasmids pRO1966 and pRO1963 did not allow growth on phenol when in *trans* with pRO2342, suggesting that the *phlR* gene spans the *Clal* and *Xho*I sites at map coordinates 16.25 and 16.5 kb (Fig. 1).

To confirm the placement of *phlR* on plasmid pRO1960, the three internal *Eco*RI fragments of pRO1960 were separately subcloned into vector plasmid pRO1769. Plasmid pRO1981, which contains the 5-kb *Eco*RI fragment (map coordinates 13.25 to 18.25 kb) from pRO1960, permitted growth on phenol when in *trans* with pRO2342, whereas plasmids pRO1982 and pRO1983 did not permit growth on phenol when pRO2342 was present in *trans* (Fig. 1).

Expression and inducibility of phenol hydroxylase in *P. aeruginosa*. Phenol hydroxylase activity was detected in cell extracts of cells of *P. aeruginosa* PAO1c grown in phenol plus Casamino Acids when carrying plasmids pRO1957 or pRO1959. The activity levels were comparable to those found in phenol-induced cells of *P. pickettii* PKO1 (Table 2). Fully induced levels of phenol hydroxylase were also found in cells of *P. aeruginosa* PAO1c that carried pRO2342 in *trans* with either pRO1960 or pRO1981.

In addition to phenol, the three isomers of cresol were tested for their ability to induce phenol hydroxylase activity in cells of *P. aeruginosa* PAO1.93 carrying both pRO2342 and pRO1981. The results presented in Table 3 show that phenol hydroxylase was induced by phenol or *m*-cresol but not by *o*-cresol or *p*-cresol. However, cells grown on either

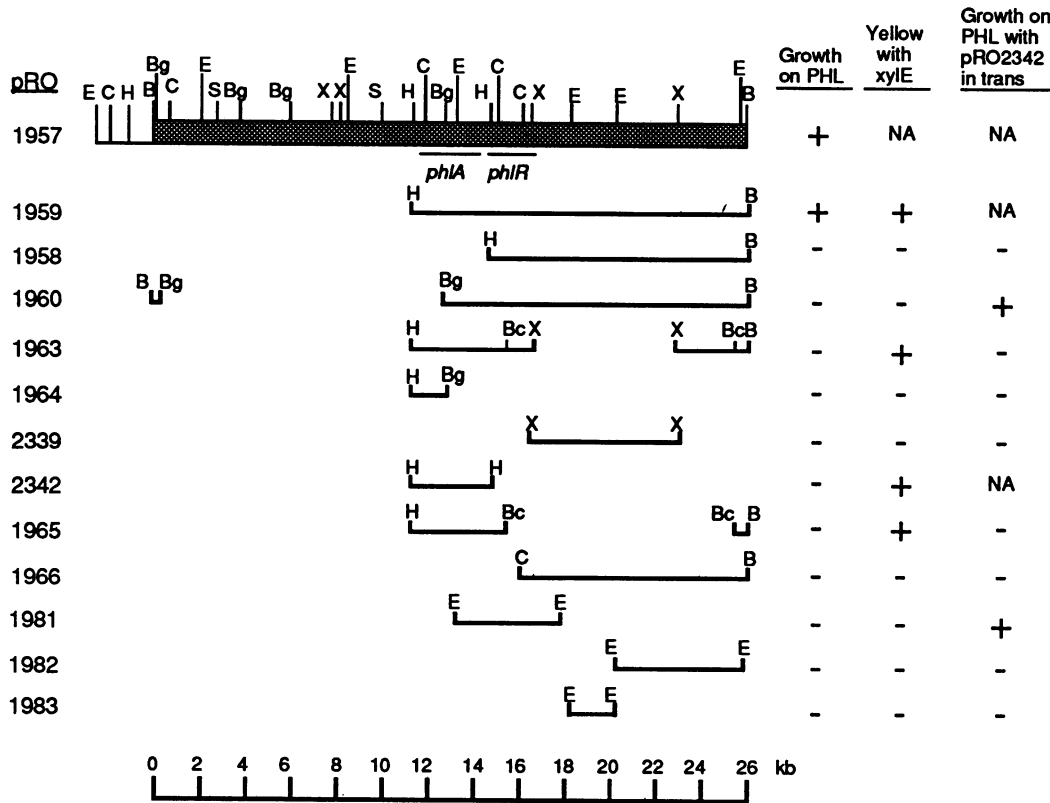


FIG. 1. Restriction endonuclease map of a *P. pickettii* PKO1 chromosomal DNA fragment, and subclones derived therefrom, which allows growth on phenol in *P. aeruginosa* PAO1c. Details of the plasmid constructions are provided in the text and in Table 1. The ability (+) or inability (-) of the plasmids to (i) allow PAO1c to grow on phenol minimal medium (Growth on PHL), (ii) produce a yellow color when in *trans* with the TOL plasmid *xylE* gene (Yellow with *xylE*), or (iii) allow PAO1c to grow on phenol minimal medium when pRO2342 was present in *trans* (Growth on PHL with pRO2342 in *trans*) is indicated to the right of the restriction maps. The positions of relevant restriction sites from plasmid cloning vectors are shown flanking pRO1957. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; S, *Sst*I; X, *Xho*I; H, *Hind*III; Bc, *Bcl*I; NA, not applicable.

inducing substrate were able to hydroxylate all the other substrates (see below). Catechol and methylcatechols were not inducers of phenol hydroxylase (Table 3).

Detection of *phlA* gene product. Plasmid-encoded proteins from pRO1959 were synthesized in *P. aeruginosa* PAO1.93, a *cata* mutant, grown on 5 mM phenol plus 0.3% Casamino Acids. The 100,000 × *g*-cleared supernatant solutions from cell extracts were separated by gel electrophoresis on an SDS-7% polyacrylamide gel (Fig. 2) and stained with

Coomassie blue. A band with an apparent *M_r* of 80,000 was present in the extract of phenol-induced cells carrying pRO1959 but was only barely detectable in the extract of Casamino Acids-grown cells.

Substrate specificity. Phenol, *o*-cresol, *m*-cresol, and *p*-cresol were substrates for phenol hydroxylase, as evidenced by substrate-dependent oxidation of NADPH (Table 4) as well as production of the corresponding catechols. In addition, catechol, resorcinol, and 3-chlorophenol were hydroxylated by the enzyme. Guaiacol and 3,4-dimethylphenol

TABLE 2. Enzymatic activity^a for cells grown on 5 mM phenol plus 0.3% Casamino Acids

Strain	Phenol hydroxylase activity
PKO1	0.20
PAO1(pRO1957)	0.18
PAO1(pRO1959)	0.17
PAO1(pRO1960, pRO2342)	0.19
PAO1(pRO1981, pRO2342)	0.20
PAO1(pRO1960)	<0.01
PAO1(pRO1981)	<0.01
PAO1(pRO2342)	0.01
PAO1	<0.01

^a Values are units of enzyme activity (as defined in the text) per milligram of protein. Each value represents the mean of three independent determinations.

TABLE 3. Induction of phenol hydroxylase activity^a in cells of *P. aeruginosa* PAO1.93 carrying both pRO2342 and pRO1981

Inducing substrate	Activity with the following assay substrate:			
	Phenol	<i>o</i> -Cresol	<i>m</i> -Cresol	<i>p</i> -Cresol
Phenol	0.17	0.14	0.25	0.17
<i>o</i> -Cresol	0.02	0.03	0.03	0.02
<i>m</i> -Cresol	0.15	0.12	0.22	0.15
<i>p</i> -Cresol	0.01	0.02	0.01	0.01
Catechol	0.01	0.02	0.01	0.01
3-Methylcatechol	0.02	0.01	0.01	0.02
4-Methylcatechol	0.01	0.03	0.02	0.02

^a Values are units of enzyme activity (as defined in the text) per milligram of protein. Cells were grown in 0.3% Casamino Acids with 0.05% inducing substrate. Phenol hydroxylase activity for cells grown in 0.3% Casamino Acids alone was 0.01 U/mg of protein.

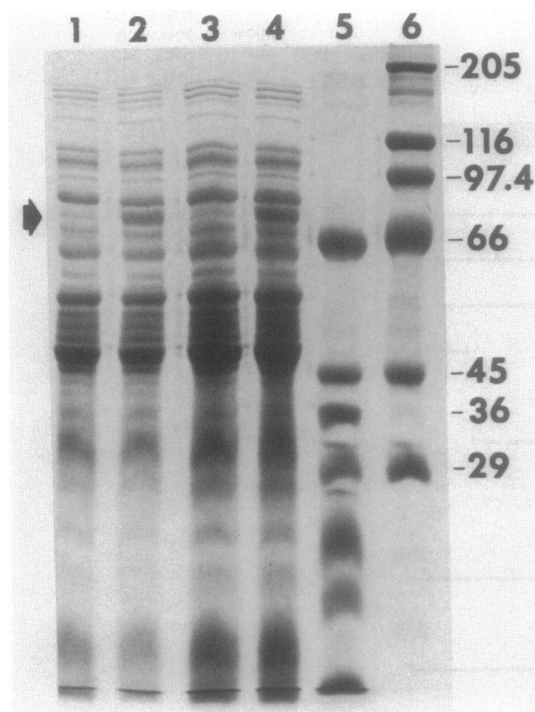


FIG. 2. SDS-polyacrylamide gel electrophoretic profile of $100,000 \times g$ -cleared soluble cellular proteins of *P. aeruginosa* PAO1.93 carrying pRO1959, grown on 0.3% Casamino Acids plus 5 mM phenol (lanes 2 and 4) or 0.3% Casamino Acids (lanes 1 and 3). Lanes 3 and 4 were loaded with twice as much protein as in lanes 1 and 2. Sizes of molecular weight markers ($\times 10^3$) (lanes 5 and 6) are shown to the right. The arrow indicates the position of the putative phenol hydroxylase.

stimulated NADPH oxidation in the presence of the enzyme (Table 4); however, no products were detected in HPLC analysis of the reaction mixture. Hence, these compounds appeared to behave as pseudosubstrates (designated nonsubstrate effectors by Massey and Hemmerich [26]). The following compounds did not promote a substrate-dependent oxidation of NADPH: 2,4-dichlorophenol, 2-chloro-5-methylphenol, 4-chloro-3-methylphenol, 2,3-dimethylphenol, 2,4-dimethylphenol, 2,5-dimethylphenol, 2,6-dimethylphenol, 3,5-dimethylphenol, *p*-hydroxybenzoate, salicylate, *m*-hydroxybenzoate, and hydroquinone.

TABLE 4. Substrate specificity of phenol hydroxylase from cells of *P. aeruginosa* PAO1.93 carrying both pRO2342 and pRO1981

Aromatic compound	Phenol hydroxylase activity ^a
True substrates	
Phenol.....	100
<i>o</i> -Cresol.....	81
<i>m</i> -Cresol.....	144
<i>p</i> -Cresol.....	100
Resorcinol.....	39
Catechol.....	14
3-Chlorophenol.....	11
Nonsubstrate effectors	
3,4-Dimethylphenol.....	69
Guaiacol.....	12

^a Activities are given as a percentage of the activity toward phenol, which was 0.17 U/mg of protein.

The product obtained from hydroxylation of phenol was identified as catechol, based on HPLC analysis (3.6-min retention time) and UV absorption spectrum. 3-Methylcatechol (5.3-min HPLC retention time) was produced from hydroxylation of *o*-cresol or *m*-cresol, whereas 4-methylcatechol (4.8-min HPLC retention time) was produced from *p*-cresol. Hydroxylation of catechol or resorcinol yielded pyrogallol (2.8-min HPLC retention time) as a major product. 4-Chlorocatechol (4.0-min HPLC retention time) was obtained from 3-chlorophenol.

Effects of inhibitors. Phenol hydroxylase was severely inhibited by cupric, mercuric, and silver ions, even at low concentrations (10 μ M). Ferrous sulfate had a slightly inhibitory effect at 100 μ M. The iron and copper chelators *o*-phenanthroline and diethyldithiocarbamate had little effect on activity. The heme inhibitor potassium cyanide had only a marginal effect. The enzyme was sensitive to the thiol-inhibiting reagent *p*-chloromercuribenzoate; however, this inhibition was completely reversed by stoichiometrically equivalent amounts of dithiothreitol. Dithiothreitol alone did not have a stimulatory effect on enzymatic activity.

DISCUSSION

Phenol hydroxylase from *P. pickettii* PKO1 exhibited a broad substrate specificity, with the three isomers of cresol being utilized at levels of activity comparable to that found for phenol. The enzyme also accommodated 3-chlorophenol, resorcinol, and catechol as substrates, although at very low levels of activity. The breadth of substrate specificity found for the PKO1 phenol hydroxylase is similar to that reported for the enzyme from the yeast *T. cutaneum*. Phenol hydroxylase from *P. pickettii* PKO1 appears to be similar to the yeast enzyme in several other respects. It can be concluded from inhibitor studies that the enzyme does not contain heme, nonheme iron, or copper; however, sulfhydryl groups appear to be essential for activity. As has been found for other phenol hydroxylases (4, 28), some aromatic substrates stimulated oxidation of pyridine nucleotide without undergoing hydroxylation. Massey and Hemmerich (26) have used the term nonsubstrate effector to describe such compounds. In our studies, 3,4-dimethylphenol and guaiacol stimulated NADPH consumption, but no *o*-diols were produced and the substrates could be recovered unchanged from the reaction mixture.

It is interesting that phenol hydroxylase from *P. pickettii* PKO1 exhibited its greatest activity against *m*-cresol (Table 4). Cresols have been shown to be intermediates in bacterial catabolism of toluene in *Pseudomonas mendocina* KR (12) and *Pseudomonas cepacia* G4 (34). In this context, the phenol hydroxylase from PKO1, with its broad substrate specificity, could be recruited for the evolution of an expanded catabolic pathway that might involve an initial hydroxylation of toluene to *m*-cresol. Such recruitment of preexisting enzymes with relaxed substrate specificity for accommodation to a novel catabolic pathway has been previously demonstrated by us for chloromaleylacetate catabolism in strain PKO1 (23).

The broad range of substrates accommodated by *P. pickettii* PKO1 phenol hydroxylase, as described above, contrasts sharply with the narrow range of compounds that could serve as inducers of enzyme activity. Phenol and *m*-cresol were the only effective inducers of enzyme activity found in our studies. *o*-Cresol and *p*-cresol were not inducing compounds, although they could be metabolized by cells that had been previously exposed to phenol or *m*-cresol.

This induction pattern is distinctly different from that previously reported for *P. putida* U (3, 40), in which phenol as well as the three isomers of cresol all serve as functional inducers of the enzyme.

Our work on phenol hydroxylase from *P. pickettii* PKO1 indicates that expression of the enzyme is positively controlled in this strain. Evidence for positive control obtains from the noninducible response of pRO2342 in the heterogenetic background of *P. aeruginosa* PAO1c. In such a heterogenetic background, in the absence of regulation from the chromosome, a positively controlled gene would not be inducible in the absence of its activator, whereas a negatively regulated gene would be constitutive in the absence of repressor or would exhibit an inducible response to the effector when the repressor is present. Our findings on the lack of constitutive expression and the absence of an inducible response for pRO2342 suggest that the PKO1 phenol hydroxylase gene is under positive transcriptional control. Previous work on regulation of phenol hydroxylase has suggested that expression of the enzyme is also under positive transcriptional control in *P. putida* U (40) and in *Alcaligenes eutrophus* 335 (18).

Extracts of cells of *P. aeruginosa* PAO1.93 carrying plasmid pRO1959 grown in phenol plus Casamino Acids exhibited in SDS-polyacrylamide gels a single novel polypeptide with an estimated molecular mass of 80 kilodaltons (Fig. 2). Approximately 2.2 kb of DNA would be required for synthesis of a polypeptide of this size, and this is consistent with the size of the *Hind*III fragment carried on plasmid pRO2342, which is the smallest of the subclones derived from pRO1959 that gave a positive reaction for 2-hydroxy-muconate semialdehyde production from phenol (Fig. 1). The novel 80-kilodalton polypeptide was synthesized in *P. aeruginosa* by using strain PAO1.93, which is a *catA* mutant that does not synthesize enzymes for *ortho* cleavage of catechol via beta-ketoadipate (22); thus, these enzymes would not appear in our SDS-polyacrylamide gels. Hence, we conclude that the novel 80-kilodalton polypeptide produced by cells carrying plasmid pRO1959 is the phenol hydroxylase of *P. pickettii* PKO1.

Data on comparative sizes of phenol hydroxylase from bacterial sources are lacking since the enzyme has not been completely purified from procaryotes; however, the 80-kilodalton relative molecular mass determined for the *P. pickettii* PKO1 phenol hydroxylase in this study is comparable to the 74-kilodalton monomer molecular mass for phenol hydroxylase from the yeast *T. cutaneum* (28) and is in the same size range as that found for several other bacterial aromatic flavoprotein hydroxylases (14, 17, 27, 39). Plasmid-encoded dichlorophenol hydroxylases have recently been purified from several bacterial strains that are capable of degrading chlorophenoxy herbicides (4, 25), but these enzymes show no activity toward phenol.

When *P. pickettii* PKO1 is grown on phenol, enzymes for *meta*-cleavage of catechol are induced (data not shown). Similarly, phenol-grown cells of *P. aeruginosa* carrying plasmid pRO1957, which contains the 26-kb *Bam*HI DNA fragment cloned from *P. pickettii*, also have enzymes for *meta*-cleavage of catechol (data not shown), whereas cells carrying plasmid pRO1959, from which an 11-kb *Bam*HI-*Hind*III fragment has been deleted (Fig. 1), metabolize phenol via the chromosomally encoded *ortho* cleavage pathway of *P. aeruginosa*. Further work on the characterization of the *meta* pathway encoded by plasmid pRO1957 is now in progress.

Recently, reports by Shingler et al. (35) and Kivisaar et al.

(21) have indicated that enzymes for phenol catabolism are plasmid encoded in two *Pseudomonas* strains. We have observed that *P. pickettii* PKO1 carries a 75-megadalton cryptic plasmid (data not shown); however, the 26-kb *Bam*HI DNA fragment cloned from strain PKO1 as recombinant plasmid pRO1957 does not appear to have been derived from cryptic plasmid DNA. Comparative restriction endonuclease digests of cesium chloride-ethidium bromide-purified cryptic plasmid DNA show no identity of restriction fragments with those produced from digestion of the *Bam*HI insert in plasmid pRO1957. Therefore, we conclude that the *Bam*HI fragment of pRO1957 was derived from chromosomal DNA of *P. pickettii* PKO1.

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