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Complete Nucleotide Sequence of *tbuD*, the Gene Encoding Phenol/Cresol Hydroxylase from *Pseudomonas pickettii* PKO1, and Functional Analysis of the Encoded Enzyme

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The gene (*tbuD*) encoding phenol hydroxylase, the enzyme that converts cresols or phenol to the corresponding catechols, has been cloned from *Pseudomonas pickettii* PKO1 as a 26.5-kbp *Bam*HI-cleaved DNA fragment, designated pRO1957, which allowed the heterogenetic recipient *Pseudomonas aeruginosa* PAO1c to grow on phenol as the sole source of carbon. Two subclones of pRO1957 carried in *trans* have shown phenol hydroxylase activity in cell extracts of *P. aeruginosa*. The nucleotide sequence was determined for one of these subclones, a 3.1-kbp *Hin*dIII fragment, and an open reading frame that would encode a peptide of 73 kDa was found. The size of this deduced peptide is consistent with the size of a novel peptide that had been detected in extracts of phenol-induced cells of *P. aeruginosa* carrying pRO1959, a partial *Hin*dIII deletion subclone of pRO1957. Phenol hydroxylase purified from phenol-plus-Casamino Acid-grown cells of *P. aeruginosa* carrying pRO1959 has an absorbance spectrum characteristic of a simple flavoprotein; moreover, the enzyme exhibits a broad substrate range, accommodating phenol and the three isomers of cresol equally well. Sequence comparisons revealed little overall homology with other flavoprotein hydroxylases, supporting the novelty of this enzyme, although three conserved domains were apparent.

Soil bacteria, including members of the genus *Pseudomonas*, are able to degrade a wide variety of aromatic hydrocarbons. In oxygenated environments, this degradation proceeds via enzymatic mono- or dihydroxylation of the aromatic nucleus, leading to *ortho*-dihydroxy-substituted products (catechols) which are the substrates for ring cleavage and subsequent entry into central metabolism.

We have previously reported (11) on the isolation and characterization of *Pseudomonas pickettii* PKO1, a strain that metabolizes benzene and toluene via phenolic intermediates (phenol and *m*-cresol, respectively) that are further hydroxylated to catechol or methylcatechols prior to *meta*-ring cleavage. The genetic material carrying this toluene- and benzene-degradative capability was cloned from the chromosome of *P. pickettii* PKO1 as a 26.5-kbp DNA fragment designated pRO1957. The individual genes encoding these degradative enzymes have been mapped, and the genes have been expressed in *Pseudomonas aeruginosa* PAO1 (24).

The phenol/cresol hydroxylase-encoding structural gene, *tbuD*, was subcloned from pRO1957 as a 3.1-kbp *Hin*dIII fragment (25). We report here the nucleotide sequence of a 2,019-bp region of the 3.1-kbp DNA fragment that carries *tbuD*. The peptide encoded by *tbuD* was purified and shown to be a flavoprotein capable of hydroxylating phenol as well as a broad range of substituted phenols.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. aeruginosa* PAO1 (15) and *Escherichia coli* DH5α (13), used for construction and maintenance of plasmids, were cultured at 37°C on plate count complex medium (36) or Luria-Bertani medium (42), respectively. Plasmids were introduced into *E. coli* by the procedure of Hanahan (13) and into *P. aeruginosa* by the procedure of Mercer and Loutit (31). Ampicillin at 100 μg/ml and carbenicillin at 500 μg/ml were used for selection of plasmid-encoded β-lactamase in *E. coli* and *P. aeruginosa*, respectively. Isolation and purification of plasmid DNA by centrifugation in cesium chloride-ethidium bromide density gradients, restriction endonuclease digestion, and molecular cloning were done as described previously (35).

Nucleotide sequence determinations. Plasmid pGEM3Z (Promega Corp., Madison, Wis.) was used to construct the subclones necessary for DNA sequencing. Ordered deletions of subclones were made by using exonuclease III and S1 nuclease as described by Henikoff (14). DNA for sequencing was routinely prepared by the method of Holmes and Quigley (16) and was further purified by passage through Plasmid Quik columns (Stratagene, La Jolla, Calif.). Nucleotide sequences were determined directly from plasmids by the dideoxy chain termination technique (43) using SP6 and T7 primers (Promega), modified T7 polymerase (Sequenase; United States Biochemical Corp., Cleveland, Ohio), and [α-^{32}P]dATP as described by Tabor and Richardson (46), except that dITP was used in place of dGTP to eliminate band compression in GC-rich regions. The resulting oligonucleotides were separated electrophoretically in 7.0 M urea–5% polyacrylamide gels in TBE buffer (0.1 M Tris, 0.089 M boric acid, 0.002 M disodium-EDTA). To eliminate secondary structure, wedge gels (0.2 to 0.6 mm thick) bound to a glass plate with γ-(methacryloxy)propyltrimethoxysilane were run at 1,500 V at a constant temperature of 60°C as described by Garoff and Ansorage (10). Following electrophoresis, the gels were fixed in 10% acetic acid for 15 min and then washed in distilled water for 15 min. Gels were dried at 80°C and exposed to X-ray film for 4 to 10 h. Nucleotide and deduced amino acid sequences were analyzed by using MacVector sequence analysis software (IBI, New Haven, Conn.).

Enzyme assays. Phenol hydroxylase activity was assayed

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by measuring the decrease in A_{340} with NADPH as the cosubstrate (32). The reaction was performed at 25°C in 1.0-ml quartz cuvettes with a 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 enzyme. 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.

Purification of phenol hydroxylase. Phenol hydroxylase was purified from cells of *P. aeruginosa* PAO1 carrying pRO1959, a subclone derived from *P. pickettii* PKO1 which we had previously shown (25) carries the *tbuD* (previously designated *phlA* [25])-encoded phenol hydroxylase. Crude extract was prepared from 20 g (wet weight) of cells grown in a minimal basal salts medium (45) with 0.3% Casamino Acids (Difco Laboratories, Detroit, Mich.) and 0.01% phenol. The buffer used during the purification was 20 mM sodium phosphate (pH 7.6) containing 7 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.001 mM flavin adenine dinucleotide (FAD) (buffer A). All steps were carried out at 4°C unless noted otherwise. Cells were broken by sonic oscillation, and cellular debris was removed by centrifugation at 100,000 × *g* for 1 h. Protamine sulfate (2% [wt/vol] in buffer A) was added dropwise with stirring to the supernatant solution to achieve a final concentration of 0.1 mg of protamine sulfate per mg of protein in the crude extract. After 30 min, the suspension was centrifuged at 100,000 × *g* for 1 h. The remainder of the purification was done with a Shimadzu LC-6A liquid chromatography system. The supernatant solution from the protamine sulfate-treated crude extract was applied at room temperature to a Bio-Gel (Bio-Rad Laboratories, Richmond, Calif.) DEAE-5-PW column (7.5 by 75 mm) equilibrated with buffer A containing 200 mM KCl. The column was washed with starting buffer and was then eluted with a 200 to 400 mM linear KCl gradient. Fractions (1 ml) were collected on ice and assayed for phenol hydroxylase activity. Active fractions were pooled, brought to 1.7 M ammonium sulfate, and then applied at room temperature to an HRLC MP7 hydrophobic interaction column (7.8 by 50 mm; Bio-Rad) equilibrated with buffer A containing 1.7 M ammonium sulfate. This column was washed with the equilibration buffer, and bound proteins were then eluted with a linear gradient of 1.7 to 0 M ammonium sulfate. Fractions (1 ml) were collected on ice and assayed for phenol hydroxylase activity. Activity was found in yellow fractions that eluted near the end of the gradient. Active fractions were combined, concentrated to 10 ml in an Amicon stirred cell (Amicon Corp., Danvers, Mass.), and then applied at room temperature to a Bio-Sil SEC 250 column (7.8 by 300 mm; Bio-Rad) equilibrated with buffer A. The column was eluted with this buffer, and fractions with phenol hydroxylase activity were combined and concentrated. Analysis by denaturing gel electrophoresis revealed that this preparation was not homogeneous; therefore the combined fractions from the gel filtration step were exchanged into 50 mM Tris-acetate (pH 8.5) on a G-25 column (PD-10 column; Pharmacia LKB Biotechnology, Piscataway, N.J.), and this preparation was applied at room temperature to a Bio-Gel DEAE MA7P column (7.8 by 50 mm; Bio-Rad) equilibrated with the same buffer. The column was eluted with a 0 to 500 mM KCl gradient, and enzyme activity was detected in a large, single, symmetrical peak which was resolved from the minor peaks. The active fractions were combined and concentrated, and the buffer was exchanged by chromatography on a PD-10 column equilibrated with buffer A containing 20% glycerol. The enzyme was stored in aliquots at −70°C until further use.

Analytical methods. Denaturing gel electrophoresis was performed on sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli (27). Samples were boiled for 5 min in solubilization buffer (64 mM β-mercaptoethanol, 2% SDS, 0.4 mM phenylmethylsulfonyl fluoride, 12.5% glycerol, 0.05% bromphenol blue in 10 mM Tris, pH 6.8). Gels were run for 30 min at 25 mA through a 4% acrylamide stacking gel and for a further 5 h at 20 mA through either a 10 or 8% acrylamide separating gel. Protein standards used for molecular mass estimation and their approximate molecular masses in kilodaltons were myosin, 205; β-galactosidase, 116; phosphorylase *b*, 97.4; bovine serum albumin, 66; ovalbumin, 45; and carbonic anhydrase, 29. To visualize proteins, the gels were stained with Coomassie blue.

UV-visible spectra and single-wavelength measurements were determined on a Shimadzu UV-160 recording spectrophotometer. Reverse-phase high-performance liquid chromatography (HPLC) was used to identify products obtained from reaction of aromatic substrates with phenol hydroxylase. Samples were analyzed for product formation on a Shimadzu SCL-6B HPLC system using a 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.

The native molecular weight of purified phenol hydroxylase was estimated by gel filtration on a Bio-Sil SEC 250 column (7.8 by 300 mm; Bio-Rad). The column was calibrated with thyroglobulin, immunoglobulin G, ovalbumin, myoglobin, and cyanocobalamin (all supplied by Bio-Rad) as standards. Protein was determined by the method of Bradford (4) with bovine serum albumin as the standard. NH_2-terminal amino acid sequences were determined at the University of Michigan Biomedical Research Core Facility by using an Applied Biosystems gas-phase sequencer interfaced with an on-line HPLC.

Chemicals and reagents. All of the chemicals, enzymes, and reagents used in these studies were of the highest purity commercially available and were purchased from Sigma Chemical Co. (St. Louis, Mo.), Mallinckrodt Chemical Works (St. Louis, Mo.), Helix Biotech Ltd. (Vancouver, British Columbia, Canada), or Aldrich Chemical Co. (St. Louis, Mo.).

Enzymes and reagents used for DNA manipulations were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.); Boehringer Mannheim Biochemicals (Indianapolis, Ind.); Promega Corp.; Stratagene Cloning Systems; and United States Biochemical Corp. and were used as suggested by the supplier. Sodium ampicillin was from Sigma, and disodium carbenicillin (Geopen) was from Pfizer (New York, N.Y.). Vitamin-free Casamino Acids and all other bacteriological medium components were purchased from Difco.

Nucleotide sequence accession number. The sequence data reported in this paper have been submitted to the GenBank Data Bank under accession number M98806.

RESULTS

Construction of subclones for DNA sequencing. We had previously shown (25) that the 3.1-kbp *Hin*dIII fragment of

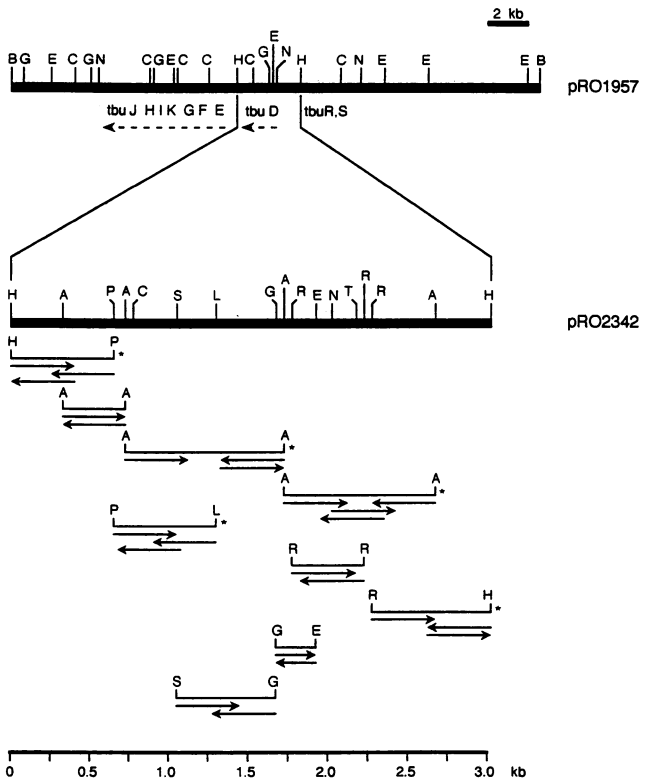

FIG. 1. Strategy used for sequencing the 3.1-kbp DNA fragment of pRO2342. The locations of *tbuD*, the *tbuEFGKIHJ* operon, and the regulatory genes *tbuR* and *tbuS* are shown on pRO1957 (25, 26) along with the directions of transcription (broken arrows) for the genes, where they are known. Solid arrows below the subclones of pRO2342 indicate the extent of the nucleotide sequence obtained from each subclone. Subclones marked with an asterisk were subjected to exonuclease III digestion as described in Results. Abbreviations: A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; L, *Sal*I; N, *Not*I; P, *Pst*I; R, *Nru*I; S, *Sph*I; T, *Stu*I.

pRO1957 (Fig. 1), which was subcloned as pRO2342, contains *tbuD*, the gene responsible for the oxidation of phenol or cresols to catechol or methylcatechols, respectively. Transfer of this *Hin*dIII fragment into the sequencing vector pGEM3Z served as the starting point for DNA sequence analysis. A series of overlapping subclones was constructed in vector pGEM3Z, and the strategy used to determine the initial sequence of these subclones is shown in Fig. 1. Each plasmid was sequenced a distance of 300 to 400 bases starting from the relevant adjacent priming site in the vector. In order to complete the sequence, a series of unidirectional deletions was made in each of the five subclones marked with an asterisk in Fig. 1. The 650-bp *Hin*dIII-*Pst*I subclone was cleaved with enzymes *Sac*I and *Bam*HI. Cleavage with *Sac*I, which has a unique site proximal to the priming region in the vector polycloning cassette, produces 3′ protruding DNA ends which are not attacked by exonuclease III, whereas cleavage with *Bam*HI, which has a unique insert-proximal site, produces 5′ protruding DNA ends which are subject to exonuclease III attack. Thus, deletion proceeds only to the left on the restriction map of the *Hin*dIII-*Pst*I subclone shown in Fig. 1. Similarly, the 650-bp *Pst*I-*Sal*I subclone was cleaved with *Sac*I and *Sal*I, with the *Sal*I-cleaved protruding DNA end subjected to exonuclease III digestion; the 1-kbp *Ava*I subclone was cleaved with *Sac*I and *Cla*I, utilizing a *Cla*I site that is internal to the cloned DNA fragment, with subsequent exonuclease III digestion of the *Cla*I-cleaved protruding DNA end; and the 750-bp *Nru*I-*Hin*dIII subclone was cleaved with *Sac*I and *Bcl*I, utilizing a unique internal *Bcl*I site, with subsequent exonuclease III digestion of the *Bcl*I-cleaved protruding DNA end. A unidirectional deletion was made from the left end of the 900-bp *Ava*I subclone by cleavage with *Sac*I and *Not*I followed by exonuclease III digestion of the *Not*I-cleaved protruding DNA end. Finally, a unidirectional deletion was made from the right end of the 900-bp *Ava*I subclone by cleavage with *Sph*I and *Bam*HI followed by exonuclease III digestion of the *Bam*HI-cleaved protruding end. Compilation of the DNA sequences from each of the derived subclones allowed for determination of the complete nucleotide sequences of the forward and reverse strands of the 3.1-kbp *Hin*dIII fragment.

Nucleotide sequence of the phenol hydroxylase-encoding region. Analysis of the nucleotide sequence of the 3.1-kbp *Hin*dIII fragment revealed a single open reading frame of 2,019 bp preceded by a putative ribosome-binding site. The nucleotide sequence of the coding strand, from the *Stu*I site to the *Hin*dIII site (map coordinates 2.2 to 0.0 kb, Fig. 1), is shown in Fig. 2 along with the translation of the open reading frame. The open reading frame is composed of 671 amino acids encoding a peptide of 73 kDa with an estimated pI of 4.76. A potential stem-loop structure (Fig. 2, nucleotides [nt] 2141 to 2172) immediately follows the termination codon for *tbuD*.

Purification and properties of phenol hydroxylase. Phenol hydroxylase was purified as described in Materials and Methods from phenol-induced cells of *P. aeruginosa* PAO1 carrying plasmid pRO1959, a partial *Hin*dIII deletant of pRO1957 which we had previously shown (25) carries *tbuD*, the structural gene encoding this enzyme, as well as its regulatory locus, *tbuR*. A summary of a typical purification procedure is given in Table 1. The enzyme obtained from such a purification was detected as a single band (M_r, 74,000) on SDS-polyacrylamide gels (Fig. 3). A native molecular weight of 147,000 was estimated from gel filtration analysis with a calibrated Bio-Sil SEC 250 column. The NH_2-terminal amino acid sequence of the purified enzyme was determined to be Thr-Lys-Tyr-Asn-Glu-Ala-Tyr-?-Asp-Val-Leu-Ile-Val-Gly-Ala-Gly-Pro-Ala-Gly-Val-Met-, which agrees with that determined by extrapolation from the nucleotide sequence (Fig. 2) of the *tbuD*-containing DNA fragment, with the initiating methionine residue being removed during maturation of the protein. The lack of an unambiguous determination for the eighth residue resulted from the inability to detect cysteinyl residues without prior chemical modification. Analysis of total protein yielding this NH_2-terminal amino acid sequence (312 pmol) accounted for all of the protein present in the sample (300 pmol); no significant (<3%) contaminating sequences were observed.

The absorbance spectrum of the purified enzyme is shown in Fig. 4. The final preparation from the purification scheme was bright yellow, and its visible spectrum had peaks at 365 and 448 nm, similar to those found in other simple flavoproteins (30). Treatment with sodium dithionite resulted in decolorization of the enzyme and disappearance of the peak at 448 nm (Fig. 4). Following reduction with dithionite, the enzyme rapidly reoxidized in air, with concomitant reappearance of the yellow color and the 448-nm peak. Flavin was released from the protein by incubating a sample (in 20 mM sodium phosphate buffer [pH 7.6]) with trichloroacetic acid (7% [wt/vol]) for 30 min and then centrifuging it to remove the resulting precipitate. When the yellow superna-

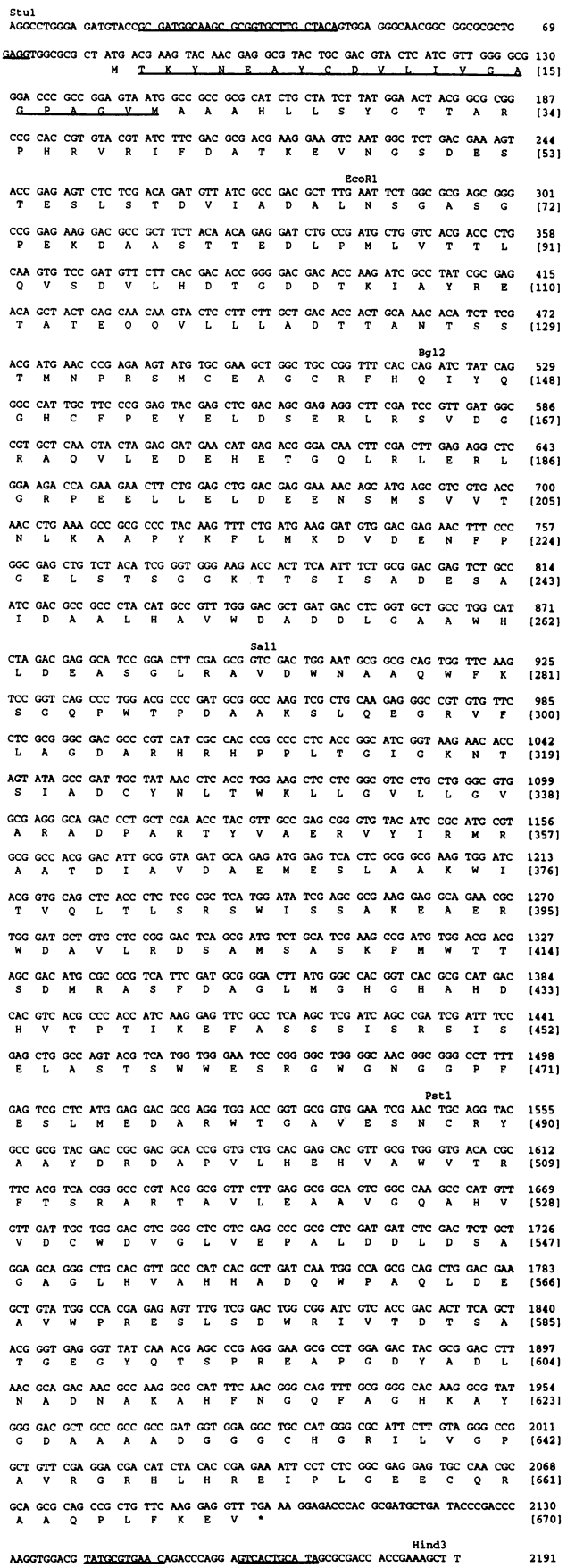

TABLE 1. Purification of phenol hydroxylase from phenol-induced *P. aeruginosa* PAO1(pRO1959)

Step	Vol (ml)	Amt of protein (mg)	Activity (U)	Sp act (U/mg)	Yield (%)
Crude extract	70	1,105	1,770	1.6	100
Protamine sulfate	77	950	1,520	1.6	86
DEAE-5-PW, pH 7.6	53	48	1,150	24	65
MP7 hydrophobic interaction	50	5	650	130	37
SEC 250 gel filtration	20	1.2	200	167	11
DEAE MA7P, pH 8.5	15	1.0	150	150	8

tant solution was analyzed by reverse-phase HPLC (1), it cochromatographed with authentic FAD rather than flavin mononucleotide. FAD was quantitated spectrophotometrically after neutralization of the supernatant solution with sodium bicarbonate. When an extinction coefficient of 11,300 M^{-1} cm^{-1} at 450 nm was used (40), the FAD content of the enzyme was determined to be 2 mol of FAD per mol of protein (M_r, 147,000).

Substrate specificity. NADPH was the preferred electron donor for phenol hydroxylase. The activity of the purified enzyme assayed in the presence of NADH was only 3% of that found when NADPH was used. There was no oxidation of reduced pyridine nucleotide in the absence of aromatic substrate.

The purified enzyme had essentially the same broad substrate specificity as was found in crude extracts (Table 2). The three isomers of cresol were as effective as phenol as substrates for the enzyme, as evidenced by substrate-dependent NADPH oxidation and production of the corresponding catechols. In addition, catechol and resorcinol were hydroxylated by the enzyme. Fluoro-, chloro-, and aminophenols were effective as substrates, with *meta*- and *para*-substituted phenols being accommodated more readily than *ortho*-substituted phenols. There was only scant activity (<1% of the activity toward phenol; data not shown) toward *meta*- and *para*-hydroxybenzoates, hydroxybenzaldehydes, and hydroxybenzyl alcohols.

The product obtained from hydroxylation of *o*-cresol by purified phenol hydroxylase was identified by HPLC analysis (5.5-min retention time) and UV absorption spectrum as 3-methylcatechol. 4-Methylcatechol (6.2-min HPLC retention time) was produced from hydroxylation of *p*-cresol. A mixture of 3- and 4-methylcatechols was obtained from hydroxylation of *m*-cresol. Hydroxylation of catechol yielded pyrogallol (3.0-min HPLC retention time), whereas benzenetriol (2.3-min HPLC retention time) was the major product obtained from resorcinol, with pyrogallol as a minor product. Fluorophenols, chlorophenols, and aminophenols were modified to more-polar substances, as evidenced by chromatographic affinity for C-18 silica columns; however, the products from these reactions were not further characterized. As found in our previous work (25), guaiacol and 3,4-dimethylphenol functioned as nonsubstrate effectors (30)

FIG. 2. Nucleotide sequence of *P. pickettii* PKO1 DNA fragment containing the *tbuD* gene. The putative promoter region (nt 19 to 45), possible ribosome-binding site (nt 70 to 73) as elucidated by Shine and Dalgarno (44), and stem-loop structure (nt 2141 to 2172 [38]) are underlined. The amino acid sequence confirmed by NH_2-terminal sequencing is underlined. Numbering in brackets for the TbuD peptide begins with the initial Thr residue.

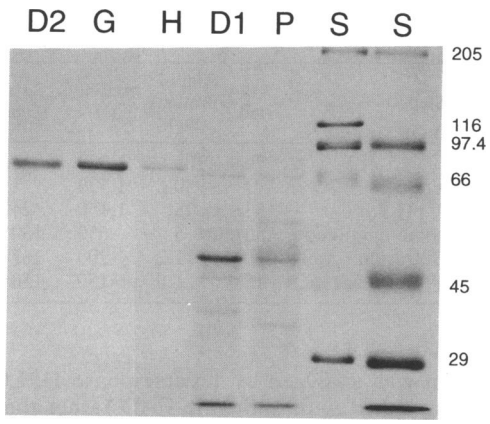

FIG. 3. SDS-polyacrylamide gel electrophoretic analysis of phenol hydroxylase from various stages of purification. Lanes: P, after protamine sulfate treatment of crude extract (135 μg of protein); D1, after Bio-Gel DEAE-5-PW ion-exchange chromatography at pH 7.6 (156 μg of protein); H, after HRLC MP7 hydrophobic interaction chromatography (5 μg of protein); G, after Bio-Sil SEC 250 gel filtration chromatography (22 μg of protein); D2, after Bio-Gel DEAE MA7P ion-exchange chromatography at pH 8.5 (12 μg of protein); S, molecular weight markers (10^3).

TABLE 2. Substrate specificity of phenol hydroxylase

Aromatic compound	Activity[a] with: Crude extract	Purified enzyme
True substrates		
o-Cresol	85	81
m-Cresol	100	110
p-Cresol	98	102
o-Fluorophenol	33	35
m-Fluorophenol	59	55
p-Fluorophenol	61	66
o-Chlorophenol	5	4
m-Chlorophenol	11	12
p-Chlorophenol	20	20
o-Aminophenol	12	5
m-Aminophenol	35	50
p-Aminophenol	55	65
Catechol	20	22
Resorcinol	35	31
Nonsubstrate effectors		
3,4-Dimethylphenol	69	60
Guaiacol	12	10

[a] Activities are given as a percentage of the activity toward phenol, which was 1.6 U/mg of protein for the crude extract and 150 U/mg of protein for the purified enzyme. Substrates were added as 5 μl of 10 mM stock solutions to the reaction mixture described in Materials and Methods.

both with the purified phenol hydroxylase and with crude extracts inasmuch as their presence in reaction mixtures stimulated NADPH oxidation without yield of more-polar substances in HPLC analysis of the reaction mixture.

The following compounds did not promote a substrate-dependent oxidation of NADPH: hydroquinone, *o*-ethylphenol, *m*-ethylphenol, *p*-ethylphenol, 2,4-dimethylphenol, 2,3-dimethylphenol, 3,5-dimethylphenol, 2,5-dimethylphenol, 2,6-dimethylphenol, salicyl alcohol, salicylaldehyde, salicylate, *o*-bromophenol, *m*-bromophenol, *p*-bromophenol, *o*-nitrophenol, *m*-nitrophenol, *p*-nitrophenol, and 4-hydroxybenzene sulfonic acid.

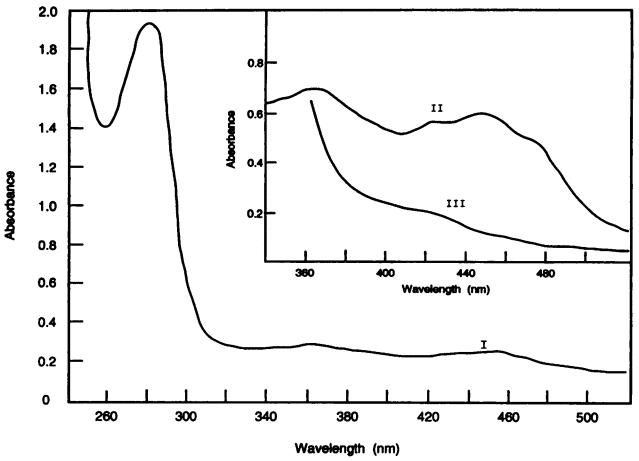

FIG. 4. Absorbance spectra of purified phenol hydroxylase. I, Enzyme in 0.05 M sodium phosphate (pH 7.6) at a protein concentration of 2.9 mg/ml; II, as in spectrum I but containing 8.7 mg of protein per ml; III, as in spectrum II after reduction with sodium dithionite.

DISCUSSION

Purified phenol hydroxylase cloned from *P. pickettii* PKO1 and expressed in *P. aeruginosa* PAO1 is a simple flavoprotein. Until now, the only microbial phenol hydroxylase available in pure form was that isolated by Neujahr and Gaal from the eukaryote *Trichosporon cutaneum* (32). Phenol hydroxylase from *T. cutaneum* and that purified from *P. pickettii* in this study are similar in several respects. Both enzymes utilize NADPH as cosubstrate in the oxidation of phenol, they are similar in their monomer molecular weights (74,000 for the peptide from *P. pickettii* and 75,000 for the peptide from *T. cutaneum*), and as native enzymes, both appear to be dimers containing 2 mol of FAD per mol of enzyme. Phenol hydroxylase from *T. cutaneum* belongs to a group of simple flavoprotein monooxygenases, best exemplified by *p*-hydroxybenzoate hydroxylase (17), which share the common oxygenation mechanism of electrophilic attack on the enzyme-bound flavin C(4a)-hydroperoxide formed as a result of aromatic ring activation owing to the presence of an electron-donating hydroxyl group on the substrate (6). Since phenol hydroxylase can now be obtained in pure form as a cloned gene product from *P. pickettii*, it will be of interest to determine whether there is a similar oxygenation mechanism for this system.

Bacterial degradation of phenol has long been studied, and much has been learned about the physiological and genetic aspects of phenol degradation, particularly among members of the genus *Pseudomonas* (2). Recent investigations of the degradation of phenol in other genera of bacteria suggest that diverse mechanisms have evolved for utilization of this substrate. A thermostable phenol hydroxylase has been partially purified from *Bacillus stearothermophilus* (12), and preliminary characterization of this enzyme suggests that it might not be a simple flavoprotein. Plasmid-encoded 2,4-dichlorophenol hydroxylase purified from *Alcaligenes eutrophus*(pJP4) (28) and *Acinetobacter* species (3) have been shown to be simple flavoproteins, but these enzymes curiously have no activity toward unsubstituted phenol. Most

recently, plasmid-encoded phenol hydroxylases from *Pseudomonas* sp. strain EST1001 (23) and *Pseudomonas* sp. strain CF600 (40) have been characterized. The former enzyme has considerable amino acid homology with the *tfdB*-encoded dichlorophenol hydroxylase from plasmid pJP4; however, it has a very narrow substrate range, accommodating only phenol and *m*-cresol (34). The latter is a multicomponent phenol hydroxylase which shares similarities with multicomponent mono- and dioxygenases involved in attack on unactivated aromatic substrates such as toluene, benzene, and naphthalene (40). It is clear from the emerging picture that there is considerable diversity in microbial mechanisms for oxidative degradation of phenol.

Phenol hydroxylase from *P. pickettii* has a substrate range similar to but somewhat broader than that found for the *T. cutaneum* enzyme. Phenol and the three isomeric meth-

A.

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TbuD   TKYNEAYC|DVLIVGAGPAGVMAAAHL|LSYGTTARPHR|VRIFD
[1]            |                   |           [42]
PhyA   TKYSESYC|DVLIVGAGPAGLMAARVL|SEYVRQKPDLK|VRIID
[1]            |                   |           [42]
TfdB     ALTIET|DVLVVGTGPAGASAGALL|ARYGVRT     |MLINK
[1]            |                   |           [36]
PheA   NETNVVET|EVLIVGSGPAGSSAAMFL|STQGISN     |IMITK
[28]           |                   |           [65]
AlkT           |AIVVVGAGTAGVNAAFWL|RQYGYKGEIR |IFSRE
[1]            |                   |           [33]
NahG   MKNNKLGL|RIGIVGGGISGVALALEL|CRYSHIQ     |VQLFE
[1]            |                   |           [38]
CamA     VNAND|NVVIVGTGLAGVEVAFGL|RASGWEGNI   |RLVGD
[1]            |                   |           [37]
TodA       AT|HVAIIGNGVGGFTTANAL|RAEGFEGRI   |SLIGD
[1]            |                   |           [34]
PobA       MKT|QVAIIGAGPSGLLLGQLL|HKAGID      |NVILE
[1]            |                   |           [32]
TbuD           |AAAADGGGCHGRILVGPA|VRGRHLHRE  |IPLGE
[626]          |                   |           [657]
               |JO-O-G-G--G---O--O|(loop)      |O-O-U
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B.

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                 *    *** * ¤ *  * ¤ ¤¤¤  ¤  ¤¤ ¤¤   ¤¤ ¤ ¤       ¤¤  ¤¤
TfdB [300] LQqGRVFcAGDAvHRHPPtnGlGsNTSIqDsfNLaWKiamVLnGtAdeslldTYtiER [357]
           ::.:::: :::: ::::::  :.: :::: : .:: ::.  :: : :       ::  ::
TbuD [294] LQEGRVFLAGDARHRHPPLTGIGKNTSIADCYNLTWKLLGVLLGVARADPARTYVAER [351]

PheA [318] LQkGRVccAGDAiHkHPPshGlGsNTSIqDsYNLcWKLacVLkGqAgpelleTYstER [375]
           :: :::  :::: :.:::  :.: :::: : ::: :::  :: : :  .   ::  ::
TbuD [294] LQEGRVFLAGDARHRHPPLTGIGKNTSIADCYNLTWKLLGVLLGVARADPARTYVAER [351]

PhyA [347] skdeRVFiAGDAcHtHsPkaGqGmNTSmmDtYNLgWKLglVLtGrAkrDilkTYeeER [404]
             . :::.:::: : : :  : : :::. : ::: :::  :: : :. :  .::  ::
TbuD [294] LQEGRVFLAGDARHRHPPLTGIGKNTSIADCYNLTWKLLGVLLGVARADPARTYVAER [351]

PobA [276] mQhGRlFLAGDAaHivPP-TG-aKglnlAas-dvs-tLyrlLLkayR-e-gRgellER [327]
           .: ::.:::::: :  :: ::  :   .:   ..   :  .::   : .  : . ::
TbuD [294] LQEGRVFLAGDARHRHPPLTGIGKNTSIADCYNLTWKLLGVLLGVARADPARTYVAER [351]

NahG [304] yvhGRVvLiGDAaHamlPhqGaGagqgleDaYfLa-rLLG [342]
             ::: : ::: :   :  : :    . : : :  .:::
TbuD [294] LQEGRVFLAGDARHRHPPLTGIGKNTSIADCYNLTWKLLG [333]
```

C.

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tbuD         GCGATGGCAAGCGCGGTGCTTGCTACA
dmpKLMNOP    ACCTTGGCACAGCCGTTGCTTGATGTC
xylCMAB      GCAATGGCATGGCGGTTGCTAGCTATA
xylS         TGCTTGGCGTTATTTTTGCTTGGAAAA
pilA         TATTTGGCATGGTAAGTGCTTGGTAGG

consensus       TGGCRNNNNNNNKTGCTWKNWR
```

FIG. 5. (A) Amino acid sequence alignments of TbuD and other ADP-binding sequences. The fingerprint ADP-binding motif proposed by Wierenga et al. (47) is shown at the bottom. J indicates H, K, N, Q, R, S, or T; O indicates A, C, I, L, M, or V; and U indicates D or E. Alignment of AlkT with CamA was published by Eggink et al. (8). You et al. (48) published an alignment of NahG with AlkT, CamA, TodA, PobA, and TfdB. Other sequences and their sources are as follows: PhyA (21), TfdB (37), PheA (34), TodA (49), and PobA (9). (B) Comparison of a portion of the deduced sequence of TbuD with sequences of other flavoprotein hydroxylases. Symbols: *, amino acid residues conserved among all of the sequences shown; ¤, amino acid residues conserved among the four phenol hydroxylases (TfdB, PheA, PhyA, and TbuD); :, residue identical with that found in TbuD; ., conservative change from the TbuD residue. Dashes have been inserted to optimize the alignment between pairs of sequences. (C) Homology of a putative *tbuD* promoter with other σ^{54} RNA polymerase-utilizing promoters. Sequences and their sources are as follows: *dmpKLMNOP* (33), *xylCMAB* (18), *xylS* (19), and *pilA* (20). R indicates A or G, N indicates any nucleotide, K indicates G or T, and W indicates T or A.

ylphenols (cresols) were equally suitable as substrates for the bacterial enzyme, whereas the yeast enzyme had only 10% of the activity with methylphenols that it had with phenol (32). We have previously reported (22) that phenol hydroxylase is part of a pathway for catabolism of benzene and toluene in *P. pickettii*, in which the unactivated aromatic hydrocarbons are initially hydroxylated to phenol or *m*-cresol, respectively.

In our previous work (25), using only crude extracts containing phenol hydroxylase, we reported that hydroxylation of *m*-cresol yielded 3-methylcatechol as product. In the present study, using the purified enzyme and an improved HPLC separation, we detected a mixture of both 3- and 4-methylcatechols as products from *m*-cresol oxidation. Such a mixture of methylcatechols would be of physiological significance for *P. pickettii* PKO1 inasmuch as our previous work on the pathway for catabolism of benzene, toluene, phenol, and cresols has shown that the *meta*-cleavage dioxygenase encoded by *tbuE* can equally well accommodate catechol, 3-methylcatechol, and 4-methylcatechol as substrate (26). Similarly, a mixture of both benzenetriol and pyrogallol, rather than just pyrogallol, which we had previously reported as the sole product, was detected as product from the oxidation of resorcinol in the present study. The possible physiological significance of the production of triols from oxidation of catechol or resorcinol by TbuD has not been elucidated.

The base composition of the 3.1-kbp *Hin*dIII DNA fragment of pRO2342 was found to be 61% G+C (data not shown), which is similar to that reported previously for other species of *Pseudomonas* (29). This high G+C value is due in large part to the codon usage preference found in the coding region for the *tbuD* gene (data not shown), where there is a strong bias (66%) toward guanine- or cytosine-terminated codons. The bias toward guanine and cytosine also extended to the variable first-position codons for leucine and arginine. This codon usage bias has been reported for many other *Pseudomonas* chromosomal genes (see reference 50 and references therein), with the notable exception of the pilin genes of *P. aeruginosa* (20), which are only about 50% G+C.

Analysis of the region upstream of the translational start of the *tbuD* gene revealed a sequence of 27 nucleotides (Fig. 2, nt 19 to 45) which showed strong homology (Fig. 5C) to a set of positively controlled promoters proposed to be transcribed by a *Pseudomonas* σ^{54}-like RNA polymerase holoenzyme (5). As shown in Fig. 5C, this putative promoter sequence for *tbuD* has considerable homology with the *xylCMAB* and *xylS* promoters of the toluene catabolic plasmid pWW0 as well as with a putative promoter sequence upstream of the *dmpKLMNOP* operon encoding the multicomponent phenol hydroxylase of *Pseudomonas* sp. strain CF600 (33). This class of promoters is recognized on the basis of a minimum canonical sequence of GG-10 bp-GC at positions −24 to −12 from the mRNA start site (7). In addition, such promoters are activator controlled, which is consistent with our previous observation (25) that expression of *tbuD* in *P. aeruginosa* was stringently dependent on the presence of a *trans*-acting locus, which we have designated *tbuR*.

The nucleotide sequence of the *tbuD* gene shows no significant overall homology with any other sequence in the GenBank or EMBL data base. However, when the deduced amino acid sequence of TbuD was compared with the sequences in the NBRF-PIR protein data base, a conserved region between residues 294 and 351 (Fig. 5B) showed significant similarity to sequences in other FAD-containing aromatic hydroxylases. The scores obtained for alignment between residues 294 and 351 of TbuD and the homologous regions of the peptides shown in Fig. 5B were as follows (a perfect match had an alignment score of 294): TfdB, 161; PheA, 146; PhyA, 144; PobA, 81; and NahG, 70. Part of this conserved region (amino acids 294 to 304 of TbuD; Fig. 5B) is composed of a short motif identified by Russel and Model (41) and later by Eggink et al. (8) as being important in FAD binding; the conserved Asp (residue 304 in TbuD; Fig. 5B), which binds the ribityl chain of the flavin moiety of FAD, is particularly important. In addition to this FAD-binding domain, 18 residues (marked by ¤ in Fig. 5B) in the deduced primary sequences are conserved among the four phenol hydroxylases.

Two additional regions of the deduced TbuD amino acid sequence showed significant homology with other flavoprotein monooxygenases: one region encompasses amino acids 9 to 42 at the amino terminus, and a second region encompasses amino acids 626 to 657 at the carboxy terminus. The amino acid sequence between residues 9 and 42 of TbuD (Fig. 5A) shows homology with a proposed consensus fingerprint sequence proposed by Wierenga et al. (47) to form a β-α-β fold which is involved in binding of the ADP moiety of FAD. This conserved motif is seen in a number of flavin-containing hydroxylases and oxidoreductases (8, 39) from disparate sources. The sequences at the amino termini of the four phenol-hydroxylating enzymes (TbuD, PhyA, TfdB, and PheA) shown in Fig. 5A align fairly well with the proposed ADP-binding fingerprint motif, with the notable exception of the initial amino acid residue of the motif, which is either Asp or Glu for phenol hydroxylases and not Lys, Arg, His, Ser, Thr, Gln, or Asn as proposed by Wierenga et al. (47). Moreover, the four phenol-hydroxylating enzymes have higher overall degrees of conservation among residues in this ADP-binding domain than in the minimum motif proposed by Wierenga et al. (47). A second potential ADP-binding fingerprint motif was detected in TbuD between residues 626 and 657 at the carboxy terminus of the deduced protein sequence (Fig. 5A). Although a functional assignment of these apparently conserved domains in TbuD must await determination of the complete three-dimensional structure of this enzyme, the fact that such regions of primary sequence are conserved among phenol hydroxylases from sources as disparate as eukaryotic yeasts, broad-host-range catabolic plasmids, and chromosomal genes from gram-negative bacteria indicates their likely functional importance to these enzymes.

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