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Catechol 2,3-Dioxygenases Functional in Oxygen-Limited (Hypoxic) Environments

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We studied the degradation of toluene for bacteria isolated from hypoxic (i.e., oxygen-limited) petroleum-contaminated aquifers and compared such strains with other toluene degraders. Three *Pseudomonas* isolates, *P. pickettii* PKO1, *Pseudomonas* sp. strain W31, and *P. fluorescens* CFS215, grew on toluene when nitrate was present as an alternate electron acceptor in hypoxic environments. We examined kinetic parameters (K_m and V_{max}) for catechol 2,3-dioxygenase (C23O), a key shared enzyme of the toluene-degradative pathway for these strains, and compared these parameters with those for the analogous enzymes from archetypal toluene-degrading pseudomonads which did not show enhanced, nitrate-dependent toluene degradation. C23O purified from strains W31, PKO1, and CFS215 had a significantly greater affinity for oxygen as well as a significantly greater rate of substrate turnover than found for the analogous enzymes from the TOL plasmid (pWW0) of *Pseudomonas putida* PaW1, from *Pseudomonas cepacia* G4, or from *P. putida* F1. Analysis of the nucleotide and deduced amino acid sequences of C23O from strain PKO1 suggests that this extradiol dioxygenase belongs to a new cluster within the subfamily of C23Os that preferentially cleave monocyclic substrates. Moreover, deletion analysis of the nucleotide sequence upstream of the translational start of the *meta*-pathway operon that contains *tbuE*, the gene that encodes the C23O of strain PKO1, allowed identification of sequences critical for regulated expression of *tbuE*, including a sequence homologous to the ANR-binding site of *Pseudomonas aeruginosa* PAO. When present in *cis*, this site enhanced expression of *tbuE* under oxygen-limited conditions. Taken together, these results suggest the occurrence of a novel group of microorganisms capable of oxygen-requiring but nitrate-enhanced degradation of benzene, toluene, ethylbenzene, and xylenes in hypoxic environments. Strain PKO1, which exemplifies this novel group of microorganisms, compensates for a low-oxygen environment by the development of an oxygen-requiring enzyme with kinetic parameters favorable to function in hypoxic environments, as well as by elevating synthesis of such an enzyme in response to oxygen limitation.

Many bacteria are resident in hypoxic aquifer environments in which dissolved oxygen is present at approximately 2 mg/liter or less (about 25% of the concentration present in air-saturated water). In three such hypoxic aquifer environments, contaminated by the aromatic petroleum hydrocarbons benzene, toluene, ethylbenzene, and xylenes (collectively designated BTEX), we demonstrated (35, 42, 45, 56) that significant microbial populations were active in situ, resulting in degradation of BTEX under oxygen-limiting conditions. In addition, the degradation of these aromatic hydrocarbons under oxygen-limiting conditions was shown to be associated with the reduction of nitrate. We have isolated bacterial strains from these hypoxic environments and have ascertained their catabolic activities over a range of environmental conditions, including denitrifying conditions and oxygen-limited environments. Three of the strains used in the present study, *Pseudomonas* sp. strain W31, *Pseudomonas pickettii* PKO1, and *Pseudomonas fluorescens* CFS215, have been isolated from aquifer sands or groundwater where significant degradation of BTEX was occurring under hypoxic conditions. In the research reported here, catechol 2,3-dioxygenase (C23O) kinetic parameters of these three representative isolates from hypoxic environments are compared with those of three archetypal aerobic BTEX degraders, *Pseudomonas putida* PaW1, *P. putida* F1, and *Pseudomonas cepacia* G4. The nucleotide and deduced amino acid sequences of the gene encoding C23O from strain PKO1, a bacterial strain representative of those capable of BTEX degradation under hypoxic conditions, are presented together with comparisons of these sequences with those of other extradiol dioxygenases. Evidence is also presented for transcriptional control, in response to substrate and also hypoxic conditions, of the *tbu meta*-pathway operon from upstream regulatory sequences. This included a sequence with homology to the recognition site for ANR, an oxygen-responsive regulatory element of *Pseudomonas aeruginosa* PAO.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Pseudomonas* sp. strain W31, *P. pickettii* PKO1, *P. fluorescens* CFS215, *P. putida* F1, *P. putida* PaW1(pWW0), *P. cepacia* G4, *Pseudomonas denitrificans*, and *Pseudomonas stutzeri* were routinely cultured on either plate count complex medium (TNA) (54) or minimal basal salts medium (BM) (42) at 30°C. For growth in the presence of volatile hydrocarbons, plates were incubated in a sealed desiccator jar which contained a small rectangle of filter paper saturated with 150 μl of the hydrocarbon.

P. aeruginosa PAO1.93 and *P. aeruginosa* PAO6261 were routinely cultured at 37°C on TNA. *P. aeruginosa* PAO4032 carrying recombinant plasmid pRO1940 and *P. aeruginosa* PAO1.93 carrying recombinant plasmid pRO1986 were cultured at 37°C on TNA with carbenicillin at 500 μg/ml.

Cells grown for determination of C23O expression under aerobic conditions were cultured in 100 ml of BM with 0.3% Casamino Acids (Difco Laboratories, Detroit, Mich.), 10 mM KNO_3, and 0.5 mM phenol with vigorous shaking in baffled 500-ml flasks. Aerobic cultures were harvested in the exponential phase (A_{425} of 0.2 to 0.3). For determination of C23O expression under oxygen-limited, denitrifying conditions, cells were grown in 100 ml of BM with 0.3% Casamino Acids, 10 mM KNO_3, and 0.5 mM phenol with slow swirling in tightly stoppered 145-ml bottles. Oxygen-limited cultures were harvested in the stationary phase (A_{425} of 0.5 to 0.6) after 18 h of incubation. The onset and extent of denitrifi-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant marker(s)[a]	Source or reference
Strains		
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 phoA hsdR17* (r_K^-, m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*	22
P. aeruginosa		
PAO1.93	*catA*	39
PAO4032	*catA met-9020 nar-9011 mtu-9002 tyu-9030*	53
PAO6261	*anr*	20, 72
P. cepacia G4	Tol^+	50
P. denitrificans		ATCC 19367 (7)
P. fluorescens CFS215	Tol^+	42
P. pickettii PKO1	Tol^+ Phl^+	19
P. putida		
PpF1	Tol^+	18
PaW1(pWW0)	Tol^+	70
Pseudomonas sp. strain W31	Tol^+	43, 44
P. stutzeri		ATCC 17588 (7)
Plasmids		
pGEM3Z	*lacZ* Ap^r; 2.75-kb cloning vector	Promega Corp.
pBluescript II KS^+	*lacZ* Ap^r; 2.96-kb cloning vector	Stratagene Cloning Systems
pRO2321	Tp^r Tc^r IncW replicon; cloning vector	77
pRO1940	Cb^r *xylS xylE*	69
pRO1957	Cb^r Tol^+ Phl^+	36
pRO1986	Cb^r *tbuWE*	37
pRO2345	Tp^r	37
pRO2347	Tp^r	37

[a] Abbreviations: Tol, toluene; Phl, phenol; Ap, ampicillin; Tp, trimethoprim; Tc, tetracycline; Cb, carbenicillin.

cation in cultures were assessed by monitoring the disappearance of nitrate and nitrite with an NI-12 colorimetric test kit (Hach Co., Loveland, Colo.). Maintenance of vector plasmid pRO2321, and recombinants based on this vector, was ensured by the addition of 600 μg of trimethoprim per ml.

Recombinant plasmids for DNA sequence analysis were maintained in *Escherichia coli* DH5α grown on Luria-Bertani medium (60) with ampicillin (100 μg/ml) at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used at concentrations of 50 and 80 μg/ml, respectively.

Hypoxic toluene utilization assays. The ability of each of the six bacterial isolates to utilize toluene under hypoxic denitrifying conditions was determined under batch incubations. Serum bottles (160-ml capacity) fitted with Teflon-lined rubber septa and aluminum crimp seals, containing 150 ml of BM and 10 ml of headspace gas, were inoculated from toluene-grown cultures of each isolate to a final A_{425} of 0.05. The medium was sparged for 20 min with argon to remove residual dissolved oxygen and then sparged a second time for 20 min with a 4.5% oxygen–95.5% nitrogen gas mixture to obtain a final concentration of 2 mg of dissolved oxygen per liter. Toluene was added to yield an effective final concentration of 30 μmol per bottle; nitrate was present at a final concentration of 10 mM. The bottles were incubated at 30°C without shaking for 48 h. Uninoculated bottles served as controls, and results were corrected for toluene losses from the controls (which were <5% of the initial toluene concentration).

Toluene concentrations were determined by reverse-phase high-performance liquid chromatography using previously described procedures (45). Determination of nitrate and nitrite concentrations was performed with the Hach NI-12 test kit.

Partial purification and assay of C23Os. C23Os were prepared from four 1-liter batches of cells grown in BM with 0.3% Casamino Acids and toluene. The toluene was supplied in the vapor phase by placing 300 μl into a small tube, which was attached to a glass rod suspended from the flask stopper. Cultures were grown to an A_{425} of 1.0 with shaking, and cells were then harvested by centrifugation. The buffer used during purification was 50 mM sodium phosphate, pH 7.5, with 10% (vol/vol) acetone (buffer A). The addition of acetone to buffer during C23O purification protected the enzyme from inactivation by oxidizing agents, as has been previously demonstrated (51). All remaining steps were carried out at 4°C unless noted otherwise. Cells were broken by sonic oscillation using multiple 15-s, 200-W bursts with a Braun-Sonic 2000 apparatus, and cellular debris was removed by centrifugation at 10,000 × *g* for 30 min. 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. Solid ammonium sulfate was added to the protamine sulfate-treated supernatant solution to achieve 32% saturation, and the pellet from this was discarded. The resulting supernatant solution was brought to 60% saturation with ammonium sulfate, and the pellet was collected by centrifugation and was dissolved in 10 ml of buffer A. The ammonium sulfate-treated extract was dialyzed overnight against two 1.5-liter changes of buffer A, and 5 ml of this extract (diluted to obtain 20 mg of protein per ml) was applied at room temperature to a Bio-Gel DEAE-5-PW column (7.5 by 75 mm; Bio-Rad Laboratories, Richmond, Calif.) attached to a Shimadzu LC-6A liquid chromatography system. The column was washed with buffer A, and proteins were then fractionated by applying a 0 to 500 mM linear KCl gradient. Fractions (1 ml) were collected on ice and were immediately assayed for C23O activity. Fractions with the greatest specific activity (which eluted at approximately 375 mM KCl) were pooled and desalted by chromatography on a PD-10 column (Pharmacia LKB Biotechnology, Piscataway, N.J.) equilibrated with buffer A containing 20% glycerol. The enzyme was stored in aliquots at −70°C until further use.

C23O of *P. putida* PaW1 was prepared from cells of *P. aeruginosa* PAO4032 carrying recombinant plasmid pRO1940, which as we have previously shown expresses the *xylE* gene (36). C23O of *P. pickettii* PKO1 was prepared from cells of *P. aeruginosa* PAO1.93 carrying recombinant plasmids pRO1986 and pRO2345 in *trans*, which as we have previously shown expresses the *tbuE* gene derived from strain PKO1 (37). All other C23Os were prepared from the native source strain.

C23O assays were performed spectrophotometrically with a Shimadzu UV-160 spectrophotometer, a thermojacketed cuvette holder, and a Haake circulating water bath by previously described procedures (4). Kinetic determinations were performed in 1.0-ml quartz cuvettes with a 1-cm light path. The final reaction volume of 1.0 ml contained 980 μl of 50 mM sodium phosphate buffer (pH 7.5), 10 μl of suitably diluted enzyme, and 10 μl of 100 mM aromatic substrate. In all cases, the reaction was initiated by the addition of the substrate.

Protein concentration was determined by the method of Bradford (9) with bovine serum albumin as the standard. Enzyme specific activities are reported as nanomoles of product produced per minute per milligram of protein.

C23O activity measurements at various oxygen concentrations. Determinations of C23O kinetic parameters at various concentrations of dissolved oxygen were performed in an anaerobic cuvette assembly (115 by 12.5 mm; model 199-QS; Hellma Cells, Inc., Jamaica, N.Y.). This cuvette assembly consisted of a 3-ml reaction cell fitted with fused duplex 1.5-ml sacs for separate addition of enzyme and substrate and a stopper, all with standard taper ground-glass joints that allowed the reaction chamber and sacs to be sealed off from the outside atmosphere. The cuvette assembly also had two gas ports permitting access to either the upper or the lower surface in the reaction chamber. The reaction buffer was sparged in the cuvette with gas mixtures of various compositions to achieve final effective dissolved oxygen concentrations ranging between 6 and 286 mM (0.19 and 9.15 mg/liter). Gases were mixed in various ratios with a Gilmont low-flow-volume GE301 rotameter. Gas mixtures that varied in percent composition of oxygen were made by mixing from compressed gas stocks of

either breathing air or analyzed custom gas mixtures that contained 5.7, 4.5, 4.0, 2.5, or 0.4% oxygen in nitrogen. Argon was used as the balance gas to achieve mixtures that varied in composition from that of the stock gases. Dissolved oxygen concentrations were determined polarographically with a Clark-style miniature oxygen electrode (model 125/05; Instech Laboratories, Plymouth Meeting, Pa.) inserted in an Instech low-volume (0.6-ml) flow chamber (model 600FH), coupled to a YSI5300 biological oxygen monitor (Yellow Springs Instruments, Yellow Springs, Ohio). All connections between the gas rotameter, the cuvette assembly, and the flowthrough oxygen electrode assembly were made with low-gas-permeability Tygon tubing (Tygon formula B-44-3; Norton Performance Plastics, Akron, Ohio).

For C23O kinetic parameter determinations, buffer was sparged in the reaction chamber of the anaerobic cuvette for 10 min, the cuvette was temporarily sealed, and then a slight positive argon gas pressure was applied to the upper gas port of the cuvette, allowing a small volume (200 to 300 μl) of buffer to pass through the Instech flow chamber for determination of dissolved oxygen content. The cuvette assembly was then sealed, the contents were mixed by several rapid inversions, and C23O activity was monitored spectrophotometrically as described above.

Molecular techniques. Plasmids were introduced into *E. coli* by the procedure of Hanahan (22) and into *P. aeruginosa* by the procedure of Mercer and Loutit (40). Restriction endonuclease digestion and molecular cloning were done as described previously (53). DNA for sequencing was routinely prepared by the method of Birnboim and Doly (6) and was further purified by passage through Qiagen tips (Qiagen Inc., Chatsworth, Calif.) as recommended by the supplier. Plasmids pGEM3Z (Promega Corp., Madison, Wis.) and pBluescript II KS$^+$ (Stratagene Cloning Systems, La Jolla, Calif.) were used to construct the subclones necessary for DNA sequencing. Ordered deletions of overlapping subclones were made by previously described procedures (38). Nucleotide sequences were determined directly from plasmids by the dideoxy chain termination technique (61) using SP6 and T7 primers (Promega) for the pGEM3Z vector or T3 and T7 primers (Stratagene) for the pBluescript vector. Sequencing reactions were performed with the modified T7 polymerase, Sequenase version 1.0, and a Sequenase kit (United States Biochemical Co., Cleveland, Ohio) as recommended by the supplier, except that dITP was used in place of dGTP to eliminate band compression in GC-rich regions.

Construction of recombinant plasmids with deletions of regions upstream of the translational start of *tbuWE* was done with the Erase-A-Base kit (Promega). Unidirectional deletions of the DNA fragment shown in Fig. 4, carried in pBluescript II KS$^+$, were made by cleavage with *Kpn*I, which has a unique site proximal to the T3 priming region in the vector polycloning cassette and which produces 3′ protruding DNA ends which are not attacked by exonuclease III, and by cleavage with *Xho*I, which has a unique insert-proximal (proximal to the *Hin*dIII site at position 1 in Fig. 4) site and which produces 5′ protruding ends which are subject to exonuclease III attack. The rate of exonuclease III digestion was controlled by performing the reactions at 17°C. Suitable deletants, confirmed by DNA sequence analysis initiated from the T3 priming site of pBluescriptII KS$^+$, were subcloned as *Pvu*II fragments into the unique *Eco*RV site within the tetracycline resistance gene of plasmid pRO2347. Plasmid pRO2347 carries a 3.9-kb *Eco*RI-*Hin*dIII DNA fragment which we have previously shown influences expression of the *tbu meta*-cleavage pathway structural genes when present in *trans* (37).

DNA sequence analyses. A complete double-stranded composite sequence was assembled from sequenced fragments with AssemblyLIGN sequence assembly software (Eastman Kodak Co., Rochester, N.Y.). Nucleotide and deduced amino acid sequences were analyzed with MacVector version 4.0 sequence analysis software (Eastman Kodak) and the University of Wisconsin Genetics Computer Group software package, version 7.3. Some of the multiple sequence alignments and phenogram analyses were performed by using LaserGene (DNASTAR, Inc., Madison, Wis.).

RNA isolation and primer extension analysis. Total RNA was isolated from cells of *P. aeruginosa* PAO1.93 carrying recombinant plasmids pRO1986 and pRO2345 in *trans*, which has previously been demonstrated to exhibit phenol-inducible expression of *tbuE* (37). Typically, 2 ml was removed from 25-ml overnight cultures, cells were harvested by centrifugation, and RNA was extracted by using Trizol Reagent (Gibco BRL, Gaithersburg, Md.) according to the manufacturer's recommended protocol, with the exception that the cells were lysed in Trizol reagent at 68°C for 10 min prior to phase separation by addition of chloroform. Precipitated nucleic acids were resuspended in 100 ml of diethyl pyrocarbonate-treated water and incubated with 10 U of RNase-free DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for 1 h at 30°C. The reaction mixtures were extracted with phenol-chloroform, and RNA was precipitated with ethanol. The precipitated RNA was dissolved in 50 μl of diethyl pyrocarbonate-treated water. The RNA concentration was determined by measuring the A_{260}. Typically, between 400 and 1,500 μg of RNA was obtained by this procedure. The quality of the RNA preparations was also assessed visually by electrophoretic analysis of a 1-μl aliquot in a 1.5% agarose gel. Electrophoresis was performed in a Tris-acetate buffer by previously described procedures (60).

The 5′ ends of the *tbuWE* transcripts were determined by primer extension analysis with primers 5′-AACATGCCCGTCAGCGAAG and 5′-CATGCGTA GTGCTCCTGG, which were complementary to nucleotides 77 to 95 (see Fig. 4, primer 1) and 44 to 61 (see Fig. 4, primer 2) downstream from the ATG translational start codon of the *tbuW* coding sequence, respectively. The primers were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase as described by Sambrook et al. (60). The oligonucleotides (2×10^5 cpm) were annealed with 50 μg of RNA in 10 μl of hybridization buffer (50 mM Tris-HCl [pH 7.7], 100 mM KCl) at 95°C for 3 min, transferred to 65°C for 5 min, and then slowly cooled to 42°C over a 30-min interval. The samples were chilled on ice, and 2 μl of 5× Superscript RT buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 2.5 mM dithiothreitol, 2.5 mM each deoxynucleoside triphosphate, and 6 U of RNasin RNase inhibitor (Promega) were added. The samples were then warmed to 42°C, and 1 μl (25 U) of Superscript RT RNase H$^-$ reverse transcriptase (Gibco BRL) was added. Extension reaction mixtures were incubated at 42°C for 1 h, after which 5 μl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF was added. The products of the extension reactions were resolved on 8% polyacrylamide gels containing 8 M urea.

Chemicals. All aromatic hydrocarbons were obtained from Aldrich Chemical Co. (Milwaukee, Wis.) and were used without further purification. Bacteriological medium components were purchased from Difco. Certified-analyzed gas mixtures containing 5.7, 4.5, 4.0, 2.5, and 0.4% oxygen in nitrogen were purchased from Liquid Carbonic Specialty Gas Corp. (Chicago, Ill.). Enzymes and reagents used for DNA manipulations were purchased from Gibco BRL, Boehringer Mannheim Biochemicals, Promega Corp., Stratagene Cloning Systems, and United States Biochemical Corp. and were used as suggested by the suppliers. Sodium ampicillin and trimethoprim were obtained from Sigma Chemical Co. (St. Louis, Mo.), and disodium carbenicillin (Geopen) was from Pfizer (New York, N.Y.).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank under accession number U20258.

RESULTS

Nitrate-dependent toluene utilization. The ability of each of the six bacterial isolates to utilize toluene under hypoxic denitrifying conditions was determined in batch incubations. This was done to compare the net effects of hypoxic conditions on an oxygen-requiring pathway. The results from such determinations, shown in Fig. 1, clearly demonstrate a nitrate-dependent enhanced degradation of toluene by *Pseudomonas* sp. strain W31, *P. fluorescens* CFS215, and *P. pickettii* PKO1 under initial conditions of hypoxia and correspond to results from preliminary work reported previously (56). In addition to the results shown in Fig. 1, the TOL plasmid pWW0 was transferred by conjugation from strain PaW1 to two archetypal denitrifying strains, *P. denitrificans* ATCC 19367 (7) and *P. stutzeri* ATCC 17588 (7). Transconjugates of *P. stutzeri* and *P. denitrificans* carrying pWW0 were able to grow on toluene as the sole carbon source when incubated under aerobic conditions; however, these transconjugates exhibited no toluene degradation when incubated under hypoxic denitrifying conditions, indicating that the toluene-degradative pathway encoded by this plasmid was not functional under hypoxic conditions in these denitrifiers (data not shown).

Determination of C23O kinetic parameters. As illustrated in Fig. 2, the six strains used in this study all catabolize toluene via a catechol or methylcatechol intermediate, which is the substrate for ring cleavage. This cleavage is catalyzed by the extradiol oxygenase C23O. We determined kinetic parameters for C23Os from each of the six strains. C23O from strain PKO1 was prepared from a cloned DNA fragment expressed in *P. aeruginosa* in order to avoid copurification of *meta*-cleavage dioxygenases from multiple pathways that might be present in this strain. The C23O of strain PaW1 was also purified from a cloned DNA fragment expressed in *P. aeruginosa*. Table 2 shows K_m determinations for oxygen (with catechol as an aromatic substrate) and for catechol and methylcatechols for the hypoxic isolates W31, PKO1, and CFS215, as well as for the nonhypoxic isolates PaW1, PpF1, and G4. Table 3 shows V_{max} determinations. On the basis of the data shown here, clearly two groupings of enzyme kinetic parameters obtain. Strains W31, CFS215, and PKO1 have Michaelis half-saturation con-

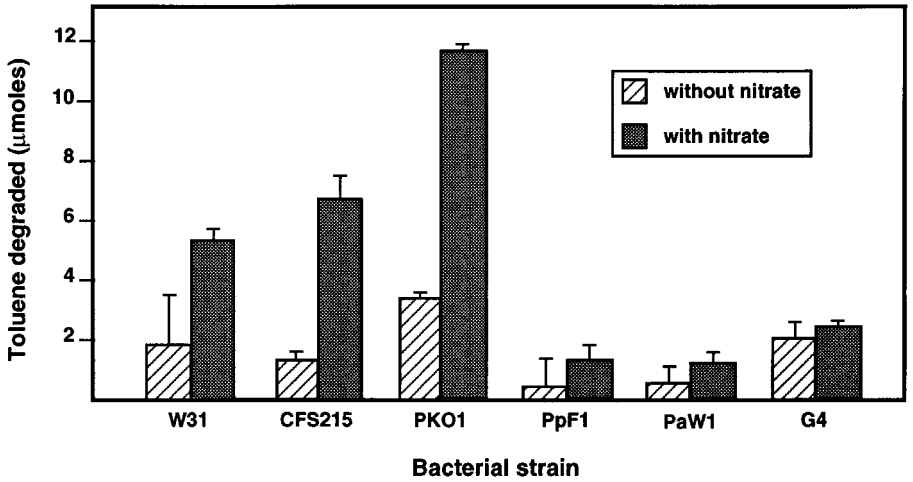


FIG. 1. Degradation of toluene in the presence and absence of nitrate following 48 h of incubation under initial conditions of hypoxia (<2 mg of dissolved O_2 per liter). Error bars indicate the standard error for duplicate determinations.

stants for oxygen and catechol substrates substantially lower than those of the nonhypoxic strains shown in Fig. 1. Similarly, this grouping of strains obtains for V_{max} results as well.

Cloning and nucleotide sequence of the *tbuE* region. The gene encoding the C23O of *P. pickettii* PKO1 was chosen for further detailed molecular analysis, as a representative oxygen-requiring enzyme from those strains able to metabolize aromatic hydrocarbons under hypoxic conditions. A 1.8-kb *Hin*dIII-*Sac*I fragment (Fig. 3), which as we had previously shown (11, 37, 55) contains *tbuE*, the gene encoding the C23O of *P. pickettii* PKO1, was subcloned from pRO1957. In the process of completing the sequence of this fragment and determining overlap with the adjacent *tbuD*-containing *Hin*dIII fragment that we had previously sequenced (38), we discovered the existence of an intervening 300-bp *Hin*dIII fragment (Fig. 3) that had not been previously mapped on pRO1957. The nucleotide sequences of both the 300-bp *Hin*dIII fragment and the 1.8-kb *Hin*dIII-*Sac*I fragment were determined. In Fig. 4 the nucleotide sequence of the coding strand of the 300-bp *Hin*dIII fragment and the adjacent fragment from the *Hin*dIII site to the *Ava*I site is shown. Analysis of the sequenced DNA revealed two open reading frames (ORF), each preceded by a Shine-Dalgarno-like sequence (62). The ORF with the translational start codon at position 238 was preceded by a putative ribo-

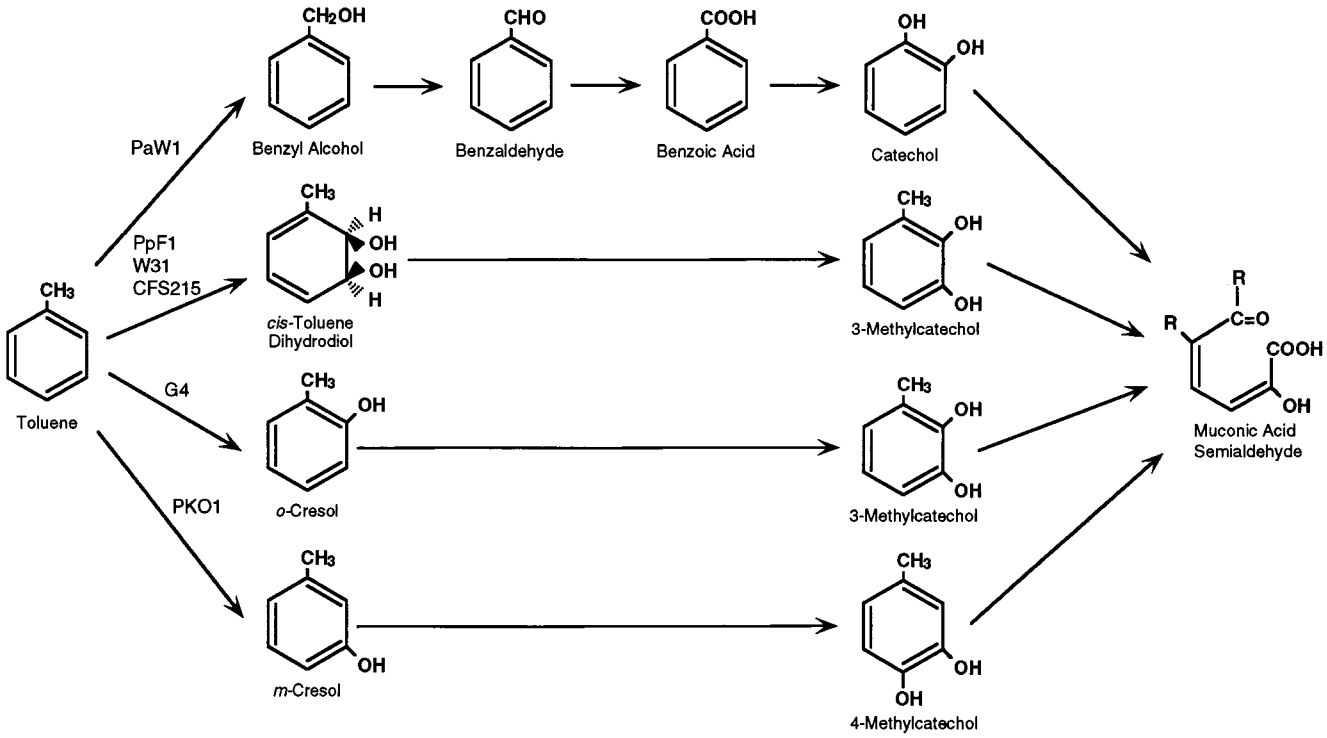


FIG. 2. Pathways for the initial catabolism of toluene described for the six *Pseudomonas* isolates used in this study.

TABLE 2. K_m determinations for C23Os from hypoxic and nonhypoxic pseudomonads

Strain	K_m [μM (SE)][a] of:			
	Oxygen	Catechol	3-Methylcatechol	4-Methylcatechol
W31	2.0 (0.06)	0.3 (0.03)	0.3 (0.06)	0.4 (0.04)
PKO1	0.7 (0.07)	0.2 (0.08)	0.2 (0.01)	0.2 (0.01)
CFS215	0.9 (0.14)	0.2 (0.02)	0.5 (0.03)	0.5 (0.03)
PaW1	3.9 (0.09)	4.1 (0.82)	4.1 (0.15)	8.2 (0.17)
PpF1	9.7 (0.06)	5.5 (0.11)	16.9 (0.38)	29.4 (0.68)
G4	6.2 (0.05)	5.1 (0.12)	1.5 (0.51)	7.8 (0.01)

[a] K_m values were estimated from Eadie-Hofstee plots, obtained from least squares regression fit to data obtained from at least three separate and independent experiments.

some-binding site, 5′-AGGGA-3′, 18 bp upstream of the ATG. This ORF was designated *tbuW*. The polypeptide sequence deduced from *tbuW* consists of 119 residues with a calculated molecular mass of 13,023 Da and a pI of 12.68. Although the function of this gene has not been established, it may be analogous in function to *xylT* of the TOL plasmid pWW0 (24, 58) as discussed below. Twenty-three nucleotides downstream of the *tbuW* stop codon was the start codon of a 945-bp ORF, designated *tbuE*. The *tbuE* gene was preceded by a putative ribosome-binding site, 5′-GGAGA-3′, 6 bp upstream of the ATG (Fig. 4). The deduced *tbuE* gene product consists of 314 amino acids, with a molecular mass of 34,576 Da and a pI of 5.99.

Sequence comparisons. The nucleotide sequence of *tbuW* has only slight overall homology with other DNA sequences in the GenBank and EMBL databases, with 9% identity found for *dmpQ* (64) and 4% identity found for *vanB* (10). Similarly, when the deduced amino acid sequence of TbuW was compared with the sequences in the SWISS-PROT and NBRF-PIR protein databases, a higher but slight degree of homology was found with a group of plant-type ferredoxins, including XylT (21% similarity) (24), NahT (19% similarity) (74), DmpQ (17% similarity), TbuC (13% similarity) (11), VanB (13% similarity), TmoF (12% similarity) (73), NahAa (12% similarity) (65), and phthalate dioxygenase reductase (10% similarity) (14).

The DNA sequence of *tbuE* has 44% identity with *tdnC*, the gene encoding 3-methyl-C23O from the aromatic amine catabolic plasmid pTDN1 (GenBank accession no. X59790) (unpublished data) and 30% identity with the gene encoding C23OII of plasmid pWW15 (GenBank accession no. U01826) (unpublished data); however, the similarity to other C23O-encoding genes in the nucleotide databases is less than 10%. The polypeptide sequence of C23O deduced from the nucleotide sequence of *tbuE* was compared with those of 27 other extradiol dioxygenases. The phenogram of the similarity among these sequences (Fig. 5) clearly shows two large subfamilies within the extradiol dioxygenases. As has been previously suggested (2, 25, 29), these subfamilies largely correspond to the structure of the substrates accommodated by the enzymes, with the upper half of the dendrogram grouping enzymes that cleave monocyclic substrates and the lower half of the dendrogram grouping enzymes that primarily cleave bicyclic substrates. The 3-methyl-C23O encoded by *todE* of *P. putida* F1 is an exception to this pattern inasmuch as it preferentially cleaves a monocyclic substrate; however, it has been demonstrated that this enzyme can accommodate 2,3-dihydroxybiphenyl as a substrate (76). Within the subfamily of enzymes preferentially accommodating monocyclic substrates, three clusters can be recognized. The first cluster includes TbuE from *P. pickettii* PKO1, which is described here, as well as TdnC (GenBank accession no. X59790) (unpublished data) and C23OII (GenBank accession no. U01826) (unpublished data). The second and largest cluster comprises XylE of plasmid pWW0, BztE, BphE, NahH, PhlH, DmpB, PhhB, C23O of strain KF711, XylE of plasmid pDK1, XylE of strain B1, and CmpE. The third cluster includes PheB, Cdo, and MndD, which are found in three gram-positive organisms. The C23O encoded by *mpcII* of *Alcaligenes eutrophus* JMP222, although apparently functionally similar to the monocyclic extradiol dioxygenases, is only distantly related to this subfamily of enzymes on the basis of overall phenetic similarity.

An alignment of TbuE with the 16 other extradiol dioxygenases that compose the subfamily of enzymes cleaving monocyclic substrates is presented in Fig. 6. The alignment shows that TbuE from *P. pickettii* PKO1 has a primary structure similar to those of other C23Os, with conservation of 19 amino acid residues. Of these conserved residues, His-176, Leu-203, His-232, His-251, Tyr-293, Pro-297, and Glu-303 are perfectly conserved among all of the extradiol dioxygenases from both the monocyclic and bicyclic subfamilies of enzymes presented in Fig. 5.

Functional analysis of the *tbuWE* promoter region. In order to identify the in vivo transcriptional start of the *tbu meta*-pathway operon transcript, primer extension analysis was performed with RNA isolated from cells of *P. aeruginosa* PAO1.93 carrying recombinant plasmids pRO1986 and pRO2345 in *trans*, which has previously been demonstrated to exhibit phenol-inducible expression of the *tbuE* gene from strain PKO1 (37). Two oligonucleotides, designated primers 1 and 2, located at nucleotides 77 to 95 and 44 to 61 (Fig. 4, boxed residues) downstream from the ATG translational start codon of the *tbuW* coding sequence were used. A single band corresponding to an A residue 24 bp upstream of the ATG codon of *tbuW* was detected with both primers (Fig. 7). These results place the start of the operon transcript at a position consistent with initiation of transcription from a putative −24, −12 promoter (Fig. 8), as discussed below.

Analysis of the region upstream of the translational start codon of the *tbuW* gene revealed sequences (Fig. 8, positions 165 to 178, boxed residues) which had homology with promoters recognized by σ^{54}-containing RNA polymerase (41). This type of promoter is minimally characterized by a GG-N_{10}-GC canonical sequence at positions −24 and −12 from the mRNA start site. Upstream of the putative σ^{54} promoter, at positions 103 to 108 and 114 to 119 (Fig. 8, short arrows) was a short perfect inverted repeat, and at positions 23 to 37 and 49 to 63 (Fig. 8, long arrows) was a longer imperfect inverted repeat. In

TABLE 3. V_{max} determinations for C23Os from hypoxic and nonhypoxic pseudomonads

Strain	V_{max} [nM min^{-1} mg of $protein^{-1}$ (SE)][a] of:			
	Oxygen	Catechol	3-Methylcatechol	4-Methylcatechol
W31	293 (24)	409 (30)	125 (13)	208 (8)
PKO1	110 (6)	847 (22)	560 (34)	108 (5)
CFS215	400 (21)	321 (19)	180 (15)	273 (21)
PaW1	31 (3)	25 (2)	13 (1)	25 (2)
PpF1	43 (6)	64 (5)	17 (3)	1 (0.2)
G4	99 (11)	152 (7)	75 (4)	44 (7)

[a] V_{max} values were estimated from Eadie-Hofstee plots, obtained from least squares regression fit to data obtained from at least three separate and independent experiments.

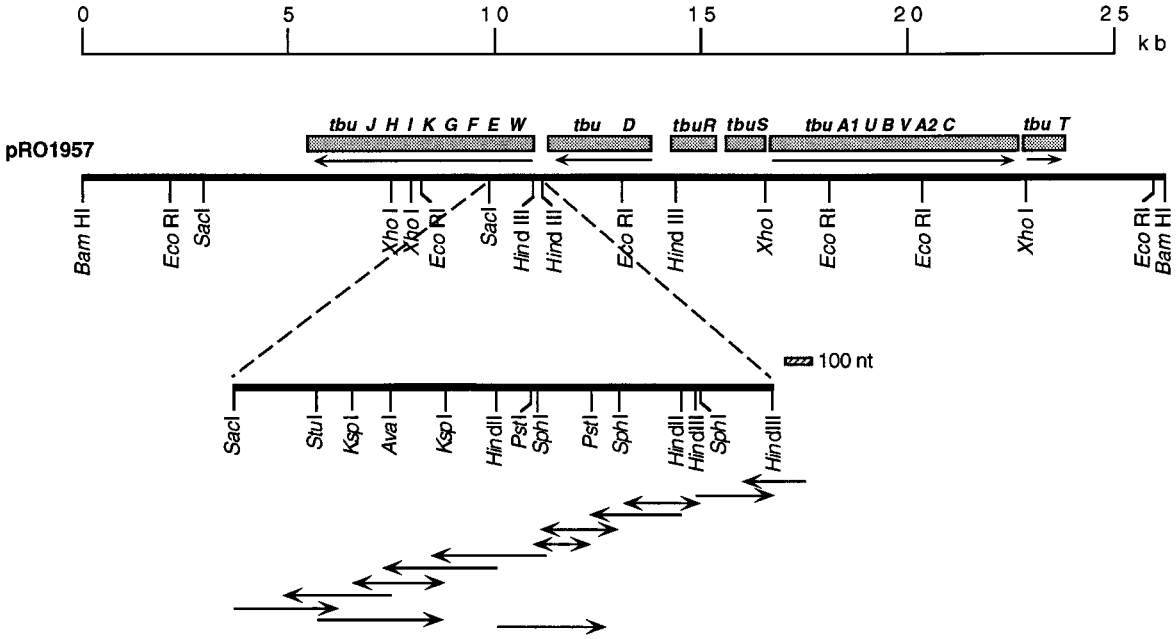


FIG. 3. Restriction map of the 26.5-kb *Bam*HI fragment cloned from *P. pickettii* PKO1 as pRO1957, showing the locations of the *tbu* genes with the direction of transcription indicated, where it is known. The *tbu* genes and their products are as follows: *tbuA1UBVA2C*, multicomponent toluene-3-monooxygenase; *tbuT*, putative regulatory gene product; *tbuR*, putative regulatory gene product; *tbuS*, putative regulatory gene product; *tbuD*, phenol/cresol hydroxylase; *tbuW*, putative ferredoxin; *tbuE*, C23O; *tbuF*, 2-hydroxymuconate semialdehyde hydrolase; *tbuG*, 2-hydroxymuconate semialdehyde dehydrogenase; *tbuK*, 4-hydroxy-2-oxovalerate aldolase; *tbuI*, 4-oxalocrotonate decarboxylase; *tbuH*, 4-oxalocrotonate isomerase; *tbuJ*, 2-hydroxypent-2,4-dienoate hydratase. The lower portion of the figure presents a detailed restriction map of the fragment sequenced in this study, along with the sequencing strategy employed. nt, nucleotides.

addition, at positions 6 to 19 (Fig. 8, double underline) we identified a sequence (TTGAC-N_4-ATCAG) identical to the recognition sequence for ANR, the anaerobic regulator of arginine deiminase and nitrate reductase in *P. aeruginosa* PAO (16).

In order to assess the functional role of sequences upstream of the *tbu meta*-pathway operon translational start on *tbuWE* expression, the deletions shown in Fig. 8 were made as described in Materials and Methods. When the entire region upstream of *tbuWE* (to the *Hin*dIII site at position 1) (Fig. 8) was present in pRO2347, a low-copy-number plasmid carrying a putative transcriptional activator of the *tbuWEFGKIHJ* operon (37), *tbuE* expression was elevated approximately twofold in denitrifying cells of *P. aeruginosa* PAO1.93 grown under oxygen-limited conditions compared with that in cells grown under aerobic conditions. This twofold increase in *tbuE* expression was absent in denitrifying cells of PAO1.93 grown under oxygen-limited conditions when the first 18 nucleotides downstream of the *Hin*dIII site (δ1 in Fig. 8) were deleted. Moreover, when *tbuWE* and the entire upstream region were present in an *anr* mutant of *P. aeruginosa*, PAO6261, no increase in *tbuE* expression was obtained when the cells were grown under oxygen-limited conditions (Fig. 8). It should be noted that the *anr* mutation in PAO6261 prevents this strain from denitrifying; thus, the growth yield attained under oxygen-limited conditions (A_{425} of 0.15 to 0.2) reflects that linked to consumption of oxygen originally present in the culture flask.

Deletion of 74 nucleotides downstream from the *Hin*dIII site (δ2 in Fig. 8) resulted in a greater than 95% reduction of *tbuE* expression. This reduction in expression was seen regardless of whether the cells were grown under aerobic or oxygen-limited conditions. Any further deletion of the region upstream of the *tbuW* translational start codon (δ3 and δ4 in Fig. 8) resulted in complete loss of *tbuE* expression. These results indicate that sequences between positions 19 and 197 (Fig. 8) are essential in *cis* for regulated expression of the *tbu meta*-pathway operon and likely comprise promoter sequences and upstream activator binding sites, as discussed below.

DISCUSSION

Kinetic analyses. The grouping of bacterial strains investigated in this study into two functional classes based upon differences in their ability to metabolize toluene, and downstream metabolites of toluene, under hypoxic conditions suggested that fundamental enzymological differences exist between these two groups of microorganisms. All six strains investigated in this study shared the common catabolic feature of the production of catechols from toluene; therefore, this common catechol intermediate, which is catalyzed by an extradiol dioxygenase with an obligatory requirement for one molecule of oxygen, was chosen for comparison of the two groups of organisms with regard to kinetic characteristics of an oxygen-requiring enzyme common to all strains.

When kinetic parameters of the extradiol dioxygenases were determined, a dramatic separation of hypoxic and nonhypoxic strains was evident. The K_m for oxygen was approximately fivefold lower for PKO1, W31, and CFS215, the bacterial strains active under hypoxic conditions, than for the other *Pseudomonas* strains, PaW1, PpF1, and G4. The hypoxic strains also have C23Os with significantly higher affinities for catechol substrates, as well as significantly higher substrate turnover rates, than are found for the nonhypoxic strains. Kinetic properties, such as K_m and V_{max}, of key catabolic enzymes can influence two of the fundamental parameters that describe the growth of a microbial cell, namely, the Monod half-saturation constant, K_s, and the maximum specific growth rate, μ_{max}. In this regard, we have also found (34) that strains PKO1, W31, and CFS215

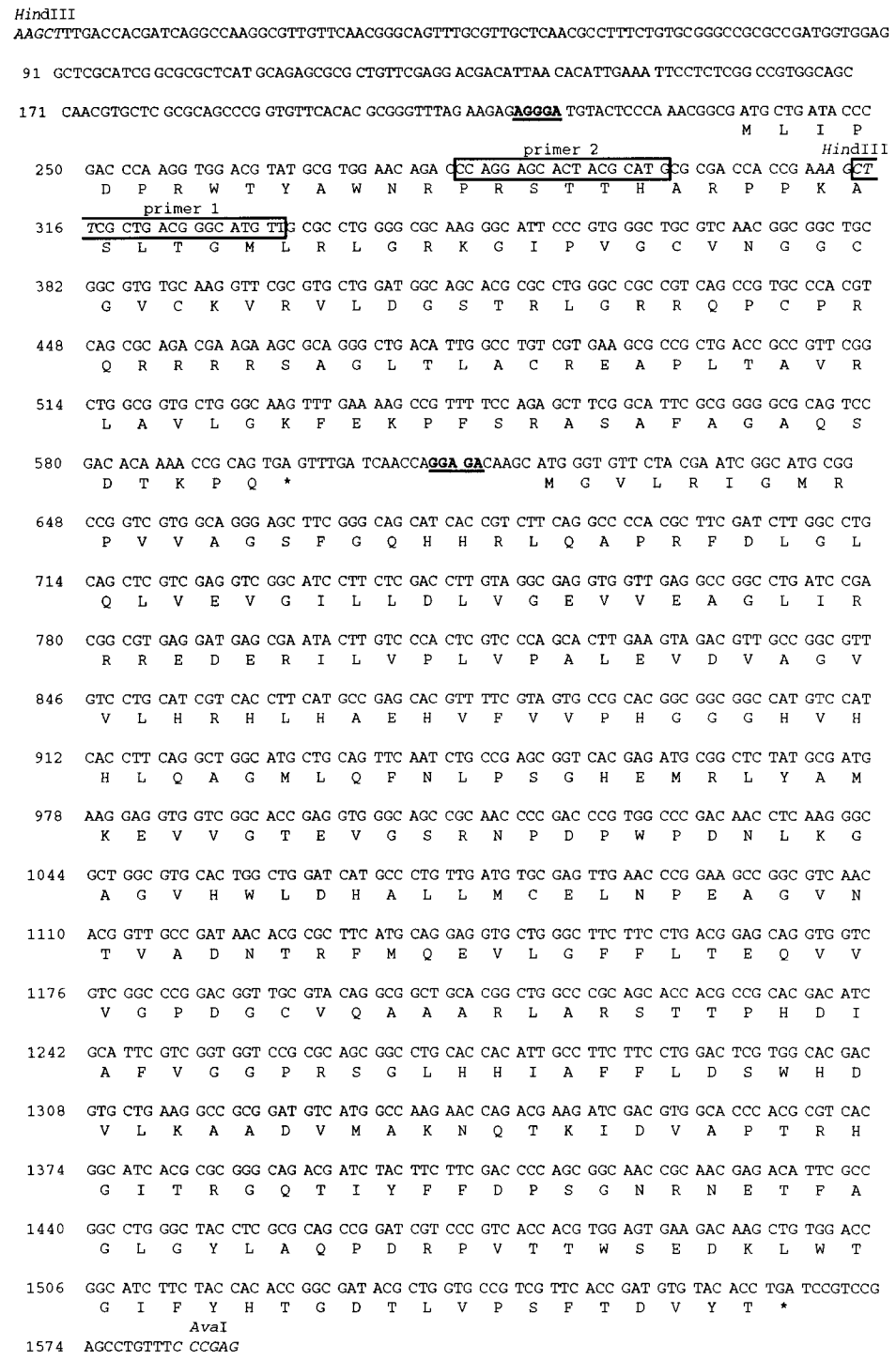


FIG. 4. Nucleotide and deduced amino acid sequences of the *tbuW*- and *tbuE*-containing DNA fragments. Putative ribosome-binding sites are underlined. The boxes designated primer 1 and primer 2 indicate nucleotides to which complementary oligonucleotide primers were made for determination of 5′ ends of the *tbuWE* transcripts, as described in Materials and Methods.

have Monod half-saturation constants for toluene that indicate an affinity for these substrates twofold greater than that found for strain PaW1, PpF1, or G4 (data not shown). Thus, it appears that the hypoxic strains are enzymatically composed to more efficiently utilize the low ambient concentrations of substrates and dissolved oxygen that occur in groundwater environments.

Sequence comparisons. As shown in Fig. 5, TbuE clearly belongs to a large family of extradiol dioxygenases in which seven residues (His-176, Leu-203, His-232, His-251, Tyr-293, Pro-297, and Glu-303) (Fig. 6) are strictly conserved. Of the seven residues that are strictly conserved among the members of this family of extradiol dioxygenases, six (four His, one Tyr, and one Glu) have been shown to be involved in active-site

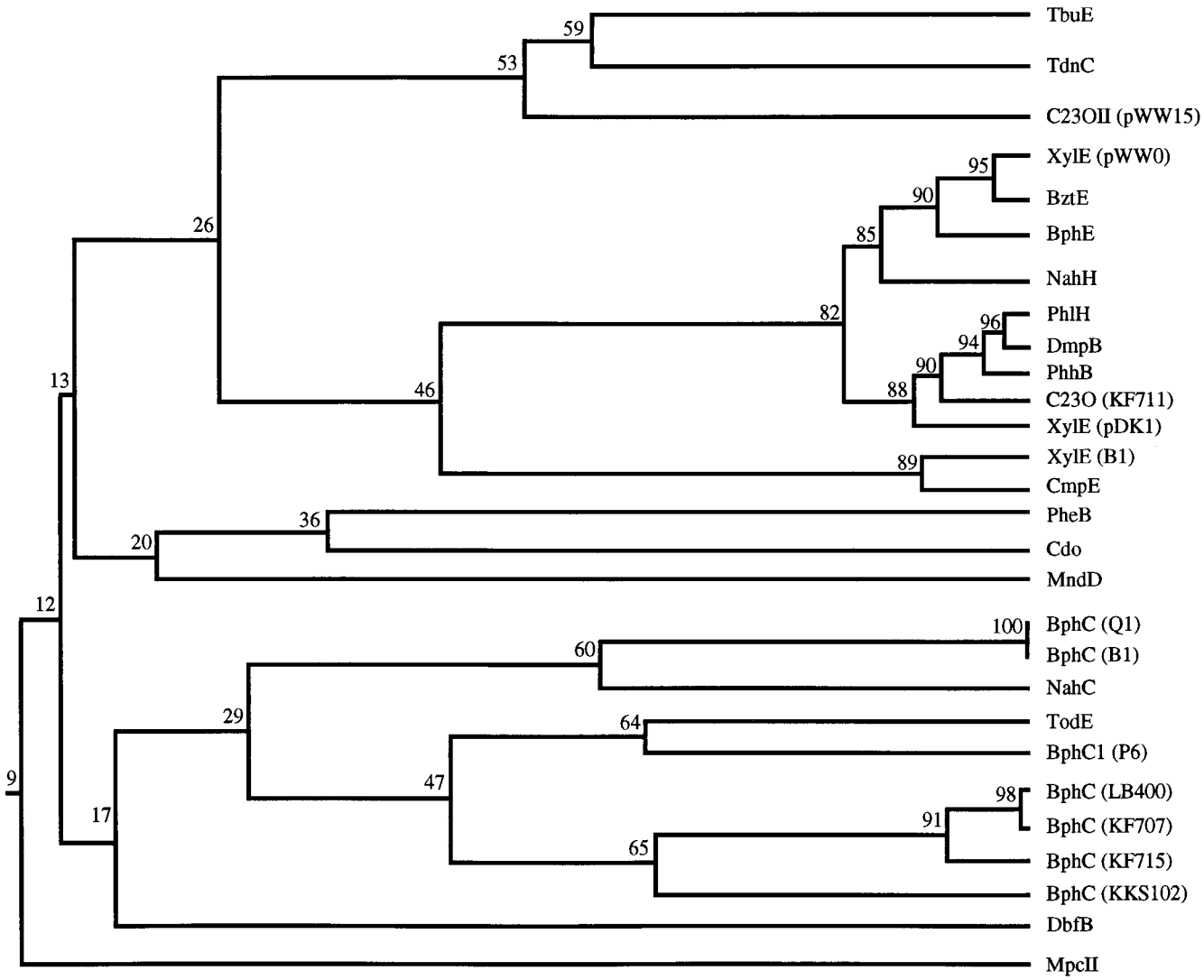


FIG. 5. Phenogram resulting from a comparison of extradiol dioxygenases. Sources of C23Os are *P. pickettii* PKO1 (TbuE), *P. putida* UCC2 (TdnC; GenBank accession no. X59790) (unpublished data), *P. putida* MT15 (C23OII; GenBank accession no. U01826) (unpublished data), *P. putida* PaW1 [XylE (pWW0)] (49), *P. aeruginosa* JI104 (BztE; GenBank accession no. X60740) (unpublished data), *Pseudomonas* sp. strain IC (BphE) (13), *P. putida* PpG7 (NahH) (17), *P. putida* H (PhlH; GenBank accession no. X80765) (unpublished data), *P. putida* CF600 (DmpB) (3), *P. putida* P35X (PhhB; GenBank accession no. X77856) (unpublished data), *Alcaligenes* sp. strain KF711 [C23O (KF711)] (47), *P. putida* HS1 [XylE (pDK1)] (5), *Beijerinckia* sp. strain B1 [XylE (B1)] (32), *Pseudomonas* sp. strain HV3 (CmpE) (75), *Bacillus stearothermophilus* FDTP-3 (PheB; GenBank accession no. X67860) (unpublished data), *R. rhodochrous* CTM (Cdo) (12), *A. globiformis* CM-2 (MndD) (8), *P. putida* F1 (TodE) (76), and *A. eutrophus* JMP222 (MpcII) (30). Sources of dihydroxybiphenyl dioxygenases are *Pseudomonas paucimobilis* Q1 (BphC) (66), *Beijerinckia* sp. strain B1 [BphC (B1)] (32), *Rhodococcus globerulus* P6 [BphC1 (P6)] (2), *Pseudomonas* sp. strain LB400 [BphC (LB400)] (29), *P. pseudoalcaligenes* KF707 [BphC (KF707)] (15), *P. putida* KF715 [BphC (KF715)] (28), and *P. putida* KKS102 [BphC (KKS102)] (33). The source of dihydroxynaphthalene dioxygenase was *P. putida* PpG7 (NahC) (25). The source of 2,2′,3-trihydroxybiphenyl dioxygenase was *Sphingomonas* sp. strain RW1 (DbfB) (23). Numbers indicate the percentage of similarity.

metal coordination in other metalloproteins (46). Histidyl residues appear to be particularly important in coordination of ferrous iron in nonheme iron enzymes, as has been shown by site-specific mutagenesis on the dihydroxybiphenyl dioxygenase of *Pseudomonas pseudoalcaligenes* KF707 (67) as well as by chemical modification of histidyl residues in the C23O of *Rhodococcus rhodochrous* CTM (12). Consistent with the foregoing observations are results from the recent determination (21) of the crystal structure of BphC, the 2,3-dihydroxybiphenyl 1,2-dioxygenase of *Pseudomonas* sp. strain LB400, which is a member of the family of extradiol dioxygenases shown in Fig. 5. Analysis of the crystal structure shows that His-176, His-241, and Glu-303 (Fig. 6) are ligands for the ferrous iron (21), and as shown in Fig. 6, these residues are conserved in TbuE as well as among all of the related extradiol dioxygenases that preferentially cleave dihydroxylated monoaromatic substrates. Further evidence in support of the importance of these residues in active-site metal coordination comes from the observations of Boldt and coworkers (8), who have shown that these potential metal ligands are also strictly conserved in MndD, a manganese-containing extradiol dioxygenase from *Arthrobacter globiformis* CM-2.

Within the subfamily of extradiol dioxygenases that preferentially cleave dihydroxylated monoaromatic substrates shown in Fig. 5, TbuE is clustered with TdnC, a 3-methylcatechol dioxygenase from an aromatic amine-degrading isolate of *P. putida*

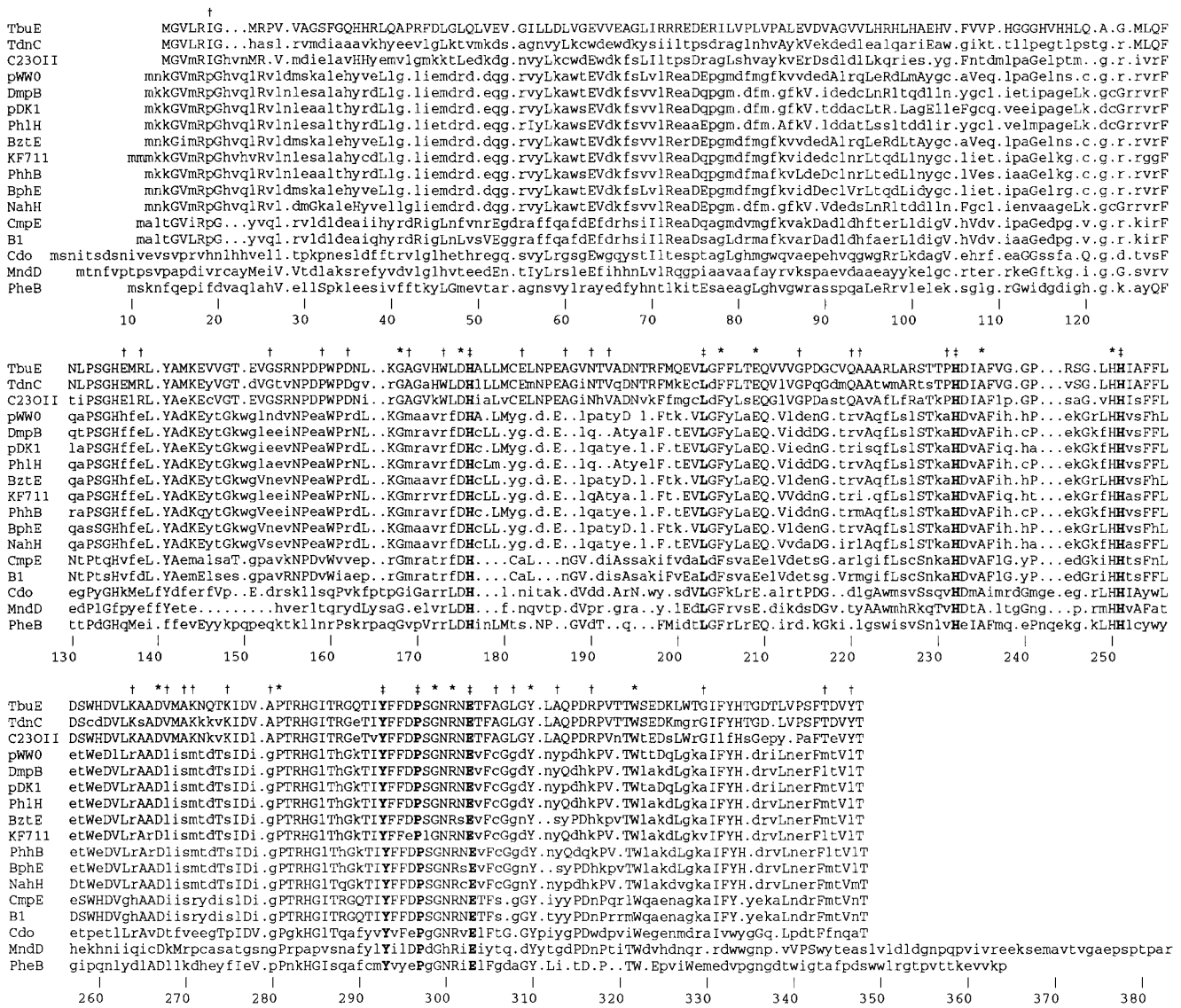


FIG. 6. Amino acid sequence alignment of TbuE and other extradiol dioxygenases that cleave dihydroxylated monoaromatic substrates. The sources of the sequences are given in the legend to Fig. 5. Residues in the compared sequences that are identical to the homologous position in TbuE are shown in uppercase. Dots were inserted to optimize the alignment. Symbols: †, amino acid residues uniquely conserved among TbuE, TdnC, and C23OII; ∗, amino acids conserved among the extradiol dioxygenases that preferentially cleave monoaromatic substrates; ‡, amino acids conserved among all extradiol dioxygenases.

(59), and C23OII, a C23O from the TOL plasmid pWW15 (31). The TbuE-TdnC-C23OII cluster of extradiol dioxygenases is united by conservation of 29 residues uniquely retained at homologous positions within the cluster (Fig. 6, residues indicated by daggers). It is reasonable to expect that conservation of amino acids unique to a cluster of enzymes might relate to unique catalytic properties among members of a cluster of monocyclic extradiol dioxygenases. In this regard, it is significant that the K_ms for catechol and methylcatechols determined for TdnC range from 0.3 to 0.4 μM (68), which are similar to the values determined for strains W31, PKO1, and CFS215 in the present study. Although comparable kinetic data are not available for C23OII, it is possible that the TbuE-TdnC-C23OII cluster of extradiol dioxygenases represents a group of enzymes that has evolved in environments that have selected for efficient utilization of dilute concentrations of substrates, as well as for catalysis at reduced oxygen concentrations. The high degree of homology among the gene products TbuE, TdnC, and C23OII appears to be independent of the phylogenetic placement of the *Pseudomonas* strain within which these genes are found, inasmuch as TbuE occurs in a *Pseudomonas* species (recently reclassified into the genus *Burkholderia* [71]) of rRNA group II (57) which is placed in the beta group of the class *Proteobacteria* (52), and TdnC and C23OII occur in a *Pseudomonas* species of rRNA group I which is placed in the gamma group of *Proteobacteria*. Thus, the evolutionary conservation among these extradiol dioxygenases suggests a common phylogenetic origin for this cluster of C23Os. Gradual modification over a long period of evolution, or parallel evolution in oxygen-depleted environments, might explain the divergence found among this cluster of C23Os. The extent of this divergence can be evaluated from inspection of the degree of substitution of the third position of synonymous codons (27). Examination of the frequencies of synonymous changes among pairwise combinations of *tbuE*, *tdnC*, and *cdo* (the gene encoding C23OII) for 77 pairs of four-codon families for conserved amino acid residues (Leu, Val, Ser, Pro,

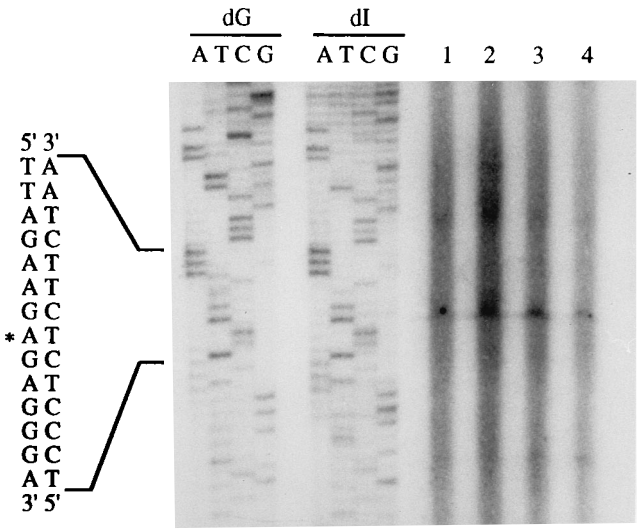


FIG. 7. Mapping of the 5′ mRNA start of the *tbu meta*-pathway operon transcript. Primer extension analysis of RNA isolated from phenol-induced (lanes 2 and 3) and uninduced (lanes 1 and 4) cells of *P. aeruginosa* PAO1.93 carrying recombinant plasmids pRO1986 and pRO2345 in *trans* was performed with [γ-^{32}P]ATP-end-labeled primer 1 (lanes 1 and 2) and primer 2 (lanes 2 and 3) as described in Materials and Methods. The reactions were run next to sequencing reactions performed on pRO1986 with primer 1 and [α-^{32}P]dATP. Sequencing reactions were performed with both dGTP and dITP reaction mixes.

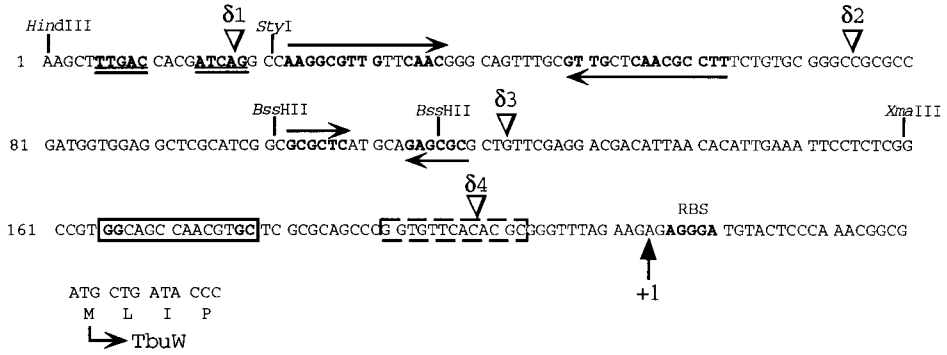
Thr, Ala, Arg, and Gly) showed that the third nucleotide was identical for 37 of the 77 codons in *tbuE* and *tdnC*, whereas third-nucleotide identity was found for only 23 and 18 of 77 codons between *cdo* and either *tbuE* or *tdnC*, respectively. Moreover, codon usage in *cdo* deviated significantly from the bias toward G or C in the wobble position that was found in *tbuE* and *tdnC* and that is frequently seen in *Pseudomonas* species and other bacterial species with a high G+C content. These results suggest that the evolution of *cdo* proceeded in parallel with that of *tbuE* and *tdnC*, but in a bacterial strain with a lower G+C content than is found in *Pseudomonas* species, and that its appearance in *P. putida* MT15 was the result of a relatively recent horizontal genetic transfer.

Concurrent with sequencing the DNA fragment carrying *tbuE* and its promoter and upstream regulatory region, we discovered a previously unmapped ORF at the beginning of the *tbu meta*-pathway operon. The translated product deduced from this ORF showed a low overall degree of similarity (<21%) to a group of proteins that possess a motif characteristic of chloroplast-type ferredoxins, including XylT (26), NahT (74), and DmpQ (64). We have designated this gene *tbuW*. Further work on characterization of the contribution of *tbuW* to the function of the *tbu meta* pathway of strain PKO1 is currently in progress. Inspection of the nucleic acid and protein sequence databases did not reveal sequences homologous to TbuW associated with C23OII of plasmid pWW15 or with TdnC, which are the extradiol dioxygenases with greatest similarity to TbuE (Fig. 5); therefore, it is not yet possible to determine whether there is overall similarity among *P. pickettii* PKO1, *P. putida* MT15, and *P. putida* UCC2 in other *meta*-pathway genes or enzymes.

Analysis of *meta*-pathway upstream sequences. The *tbu meta*-pathway transcript appears to lie downstream of a σ^{54} promoter sequence (Fig. 8). The promoters of this class conform to a precise sequence with a consensus TGGCAC-N_5-TTGCW (where W is A or T) located, in this case, at positions −26 and −11 from the mRNA start site (48). The sequence at positions 164 to 179 in the region upstream of the *tbu meta*-pathway translational start (Fig. 8, sequence enclosed in solid-line box) matches 9 of the 11 nucleotides in the consensus σ^{54} promoter sequence; however, the spacing between this putative promoter sequence and the mRNA transcript start that we identified at position 214 (Fig. 7) differs from the canonical −12, −24 placement of the promoter relative to the mRNA start that is characteristic of this class of promoters (48). A potential alternate promoter-like sequence that occurs at positions 190 to 202 in the region upstream of the *tbu meta*-pathway translational start (Fig. 8, sequence enclosed in dashed-line box) is positioned with the expected canonical −12, −24 placement with respect to the mRNA transcript start that was mapped by primer extension analysis in this study; however, this putative promoter does not conform to the consensus TGGCAC-N_5-TTGCW motif that is characteristic of this family of promoters (48). Further work is in progress to determine the contribution of these promoter-like sequences to the functionality of this region as a promoter for the *tbu meta*-pathway operon.

In addition to a conserved sequence, σ^{54}-dependent promoters are also always subject to positive control by transcriptional activators, which normally bind at least 100 bp upstream of the promoter (41). We have previously shown that induction of the *tbu meta*-pathway operon may reflect the influence of a *trans*-acting locus that we have previously designated *tbuS* (37), and on the basis of the results described in the present research, the large inverted repeat centered at 121 bp upstream of the putative promoter may be a site for binding of a regulatory gene product. Deletion of this inverted repeat reduced phenol-inducible expression of *tbuE* by 95%. Inverted repeats have been identified as activator binding sites upstream of the *xylCMABN* promoter of TOL plasmid pWW0 (1) and upstream of the *dmp* operon of *Pseudomonas* sp. strain CF600 (63), and the consensus sequence TTGNTCAA that occurs in these activator binding sites is also found as part of the dyad symmetry of the large inverted repeat upstream of the *tbu meta*-pathway operon. The smaller inverted repeat centered at 53 bp upstream of the putative promoter may also influence expression of the *tbu meta* pathway, as evidenced by the slight level of inducible expression of *tbuE* seen when this site was present. However, the precise contribution of these *cis*-acting sequences in transcriptional control of the *tbu meta*-pathway operon awaits completion of the nucleotide sequence of a putative regulatory gene and purification of the encoded protein, which is currently in progress.

Identification of a sequence upstream of the *tbu meta*-pathway promoter that was homologous to the binding site for ANR, the anaerobic regulator of arginine deiminase and nitrate reduction of *P. aeruginosa* PAO, suggested that expression of the *tbu meta*-pathway operon is influenced by oxygen-limited, denitrifying conditions. Evidence in support of such regulation was provided by experiments demonstrating a two-fold increase in *tbuE* expression in oxygen-limited cultures of *P. aeruginosa* that were carrying out significant denitrification. That this increase in *tbuE* expression was linked to denitrification and not merely to the growth phase of the culture was shown by control cultures in which cells were grown to the stationary phase in the absence of nitrate. In such cultures, no enhanced expression of *tbuE* was detected (data not shown). Moreover, deletion of the putative ANR binding sequence resulted in a loss of enhanced expression of *tbuE* for cells grown under denitrifying conditions. Taken together, these results indicate that the presence of this ANR-like binding site in *cis* clearly up-regulated the expression of the cloned *tbu meta*-pathway operon of *P. pickettii* PKO1 in *P. aeruginosa*. A variety of promoters (reviewed in references 20 and 72) in *P.*



Effects of deletions in the upstream region of the *tbu meta* pathway operon on *tbuE* expression in aerobic or oxygen limited conditions.

	C23O activity (nM min^{-1} mg^{-1} protein) [a]			
	PAO1.93		PAO6261	
plasmid	aerobic	oxygen limited	aerobic	oxygen limited
parent	722 (22)	1464 (61)	686 (37)	679 (38)
δ1	722 (42)	780 (33)	674 (38)	656 (38)
δ2	10 (0.3)	8 (0.4)	4 (0.4)	3 (0.3)
δ3	< 0.1	< 0.1	< 0.1	< 0.1
δ4	< 0.1	< 0.1	< 0.1	< 0.1

[a] Values represent the mean of determinations from at least three separate and independent experiments. Values in parenthesis are standard errors.

FIG. 8. Nucleotide sequence of the promoter and region upstream of the translational start of the *tbu meta*-pathway operon. Only the first four codons of *tbuW*, the first gene in the *tbu meta*-pathway operon, are shown. A putative ribosome-binding site (RBS) upstream of *tbuW* is indicated in boldface. A region (nucleotides 165 to 178) homologous to σ^{54}-dependent promoter consensus sequences is indicated by the solid-line box. An alternative putative σ^{54}-like promoter sequence (nucleotides 190 to 202) is indicated by the dashed-line box. The start site (+1) of the mRNA transcript mapped by primer extension analysis is designated by a vertical arrow. Inverted repeats are indicated by boldface with arrows above or below the sequences. The region homologous to the ANR consensus sequence of *P. aeruginosa* is indicated by a double underline. The extents of the four unidirectional exonuclease III deletions are indicated by inverted triangles. Recognition sites for restriction endonucleases used to map the extent of exonuclease III deletions are shown above the first nucleotide of the respective palindrome. The table presents the effects obtained from unidirectional deletions of the region upstream of the *tbuWEFGKIHJ* operon on *tbuWE* expression in cells of *P. aeruginosa* PAO1.93 or PAO6261 grown under aerobic or oxygen-limited conditions.

aeruginosa have been shown to be influenced by ANR, including those for anaerobic arginine catabolism, azurin synthesis, and nitrite reductase. In these promoters, the ANR binding site is centered at approximately −42 from the transcriptional start. This differs significantly from the placement seen upstream of the *tbu meta*-pathway operon, where the ANR-like recognition sequence is more than 100 bp upstream of the promoter and is juxtaposed with an inverted repeat that is a possible binding site for a transcriptional activator. Such an arrangement suggests that there are differences between *P. aeruginosa* and *P. pickettii* in regulation of promoters under oxygen-limited conditions and that an ANR-like homolog that functions as a transcriptional modulator exists in *P. pickettii* PKO1.

In summary, *P. pickettii* PKO1, *Pseudomonas* sp. strain W31, and *P. fluorescens* CFS215 are representative of a group of bacterial strains which, from our work to date, appear to be largely indistinguishable from closely related species by previous criteria but which have evolved a suite of adaptive traits that allow growth and oxygen metabolism in oxygen-limited environments. These adaptive traits include the ability to carry out significant nitrate-dependent degradation of aromatic hydrocarbons under conditions of oxygen limitation, the presence of key catabolic enzymes with kinetic characteristics that allow effective turnover of limiting substrates, and transcriptional enhancement of promoters of key catabolic operons linked to the onset of denitrification.

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